University of Alberta

Mechanisms Involved in the Modulation of Aryl Hydrocarbon Receptor-Regulated Genes by Inflammation

by

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ABSTRACT

Little is known about the mechanisms involved in the modulation of aryl hydrocarbon receptor (AhR)-regulated genes during pathophysiological conditions such as inflammation. The major aims of the present study were 1) to assess the effect of inflammation and the interaction between inflammation and the AhR in the regulation of AhR-regulated genes such as cytochrome P450 1a1 (Cyp1a1), glutathione S-transferase a1 (Gsta1), and NAD(P)H:quinone oxidoreductase 1 (Nqo1) in murine hepatoma Hepa 1c1c7 cells and 2) to examine the role of nitric oxide (NO) in the modulation of these genes during inflammation. Our results demonstrate that both tumor necrosis factor-α (TNF-α) and lipopolysaccharide (LPS) repressed the constitutive and inducible expression of Cyp1a1, Gsta1, and Nqo1 at mRNA and activity levels in Hepa 1c1c7 (WT), but not in AhR-deficient (C12) or ARNT-deficient (C4) cells. In addition, the induction of Gsta1 and Nqo1 mRNAs and activities by phenolic antioxidant, *tert*-butylhydroquinone (tBHQ), through the antioxidant response element (ARE) were not significantly affected by TNF-α or LPS. On the other hand, a significant increase in reactive oxygen species (ROS) was observed in WT, C12 and C4 cells treated with TNF-α or LPS which was completely prevented by tBHQ. Furthermore, various concentrations of TNF-α and LPS significantly induced NOS2 expression followed by a marked increase in NO production. Formation of NO was completely inhibited by inducible nitric oxide synthase (NOS2) inhibitor, L-N6-(1-iminoethyl) lysine (L-NIL). The down-regulation of Cyp1a1, but not Nqo1, was significantly prevented by L-NIL. However, peroxynitrite decomposer, iron tetrakis (n-methyl-4'-pyridyl) porphyrinato, (FeTMPyP)

did not alter TNF-α and LPS-mediated down-regulation of Cyp1a1 and Nqo1 at mRNA and activity levels. In conclusion, suppression of AhR-regulated genes by TNF-α and LPS is dependent on the presence of both transcription factors, AhR and ARNT. Furthermore, association of this down-regulation with increase in ROS production reveals that ROS may directly or indirectly be involved in down-regulation of AhR-regulated genes. On the other hand, NO, but not peroxynitrite, may contribute to TNF-α and LPS-mediated down-regulation of Cyp1a1 without affecting the down-regulation of Nqo1.

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LIST OF ABBREVIATIONS

AA, arachidonic acid

Act-D, actinomycin D

AHHs, aryl hydrocarbon hydroxylase activities

AhR, aryl hydrocarbon receptor

AhRR, AhR repressor

ALDH-3, aldehyde dehydrogenase-3

αNF, α-naphthoflavone

ARE, antioxidant responsive element

ARNT, aryl hydrocarbon receptor nuclear translocator

BHA, 3-*tert*-butyl-hydroxyanisole

bHLH, basic helix-loop-helix transcription factor

βNF, β-naphthoflavone

C4, ARNT-deficient Hepa 1c1c7 cells

C12, AhR-deficient Hepa 1c1c7 cells

CHX, cycloheximide

CSFs, colony-stimulating factors

CYP, cytochrome P450

DAN, 2, 3-diaminonaphthalene

EMSA, gel electrophoretic mobility shift assay

ERF, ethoxyresorufin

EROD, 7-ethoxyresorufin *O*-deethylation

FeTMPyP, iron tetrakis (N-methyl-4'-pyridyl)porphyrinato

GAPDH, glyceraldehyde-3-phosphate dehydrogenase

GSH, glutathione

GST, glutathione S-transferase

GSTA1, glutathione S-transferase A1

HAHs, halogenated aryl hydrocarbons

HBSS, Hanks' balanced salt solution

HIF, hypoxia inducible factors

HSP90, 90-kDa heat-shock proteins

H_2O_2, hydrogen peroxide

IFN, interferon

IL, interleukin

i.p., intraperitoneal

Keap1, Kelch-like ECH associating protein 1

L-NIL, L-N(6)-(iminoethyl)-lysine

LPS, lipopolysaccharide

MAPK, mitogen-activated kinases

3-MC, 3-methylcholanthrene

MRF, methoxyresorufin

MROD, 7-methoxyresorufin *O*-deethylation

MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

NAT, 2, 3-naphthotriazole

NcoA-2, nuclear coactivator-2

NF-κB, nuclear factor kappa B

NO, nitric oxide

NOS1, neuronal nitric oxide synthase

NOS2, inducible nitric oxide synthase

NOS3, endothelial nitric oxide synthase

NQO1, NAD(P)H: quinone oxidoreductase

Nrf2, nuclear factor erythroid 2-related factor-2

NFI/CTF-1, nuclear factor I/CCAAT box transcription factor

PAHs, polycyclic aromatic hydrocarbons

p/CIP, p300/CBP cointegrator protein

PKC, protein kinase C

Rb, retinoblastoma protein

ROS, reactive oxygen species

SDS, sodium dodecyl sulphate

SIM, single-minded proteins

SMRT, silencing mediator of retinoic acid and thyroid hormone receptor

SRC-1, steroid receptors-coactivator-1

SSC, saline with sodium citrate

SULT, sulfotrnasferases

tBHQ, *tert*-butylhydroquinone

tBQ, 2-*tert*-butyl(1,4)paraquinone

TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

TGF-β, transforming growth factor-β

TLR, Toll-like receptor

TNF-α, tumor necrosis factor-α

UGT, UDP-glucuronosyltransferase

UGT1A6, UDP-glucuronosyltransferase 1A6

WT, wild-type murine hepatoma Hepa 1c1c7 cells

XME, xenobiotic metabolizing enzyme

XRE, xenobiotic responsive element

With love and gratitude, I dedicate this work to:

- *My Parents and Sister who always stood by me and encouraged me.*
- *My Uncle (Dr. Jamali) and Aunt (Dr. Keshavarz) without whom this experience would have been incomplete.*

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PART I

INTRODUCTION

1. ARYL HYDROCARBON RECEPTOR

1.1. History

The function of the aryl hydrocarbon receptor (AhR) is well-known and has been studied for more than 30 years. Initially, scientists were intrigued by the extreme toxic potency of chlorinated dibenzo-*p*-dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in animals (Higginbotham et al. 1968; Schwetz et al. 1973). Later, they became fascinated by the potency of TCDD in eliciting biochemical effects (Poland and Glover 1973b; Poland and Glover 1974) such as induction of aryl hydrocarbon hydroxylase (AHH) activity (known to be catalyzed primarily by cytochrome P4501A1 (CYP1A1)), as well as by the strict structure–activity relationships for this effect (Goldstein et al. 1977; Poland and Glover 1973a; Poland and Glover 1977) and the mouse strain differences in sensitivity (Nebert et al. 1972; Poland and Glover 1974). Poland and coworkers first identified the AhR by demonstrating that radiolabeled TCDD specifically bound with high affinity to a hepatic protein from C57BL/6 mice, and this report triggered a multitude of studies on the structure, function and molecular biology of this protein, which was designated as the AhR. These observations led to the prediction and then discovery of the AhR as an 'induction receptor' that controls the expression of AHH activity (Poland and Glover 1975; Poland et al. 1976). Although originally there was a thought that AhR has a relatively narrow structural specificity (Poland and Knutson 1982), the AhR is now known to recognize a wide range of chemical structures, including non-aromatic and non-halogenated compounds (Denison and Nagy 2003).

1.2. Structure of AhR

AhR is a ligand-activated transcription factor which contains basic helix-loop-helix (bHLH) motifs near its amino-terminus (Hoffman et al. 1991). bHLH motifs are found in other transcription factors that bind specific DNA sequences as homodimers and heterodimers. Near its carboxy-terminus, the AhR contains a segment of homology that is also found in two regulatory proteins of *Drosophila melanogaster*, period (PER) and single minded proteins (SIM), and that has been termed the PER/aryl hydrocarbon receptor nuclear translocator (ARNT)/SIM (PAS) domain (Nambu et al. 1991). The PAS domain contains two copies of an approximately 50 amino acid degenerate direct segment, referred to as the PAS A and PAS B segment (Nambu et al. 1991). bHLH/PAS factors are divided to two classes: The AhR, the hypoxia inducible factors (HIF; HIF-1α, HIF-2α and HIF-3α) and SIM (SIM1 and SIM2) were categorized in class I bHLH/PAS, which in order to form active transcription factor complexes they must dimerize with a class II bHLH/PAS factor such as aryl hydrocarbon receptor translocator protein (ARNT) (Ema et al. 1996; Hoffman et al. 1991; Kewley et al. 2004; Lindebro et al. 1995; Wang et al. 1995). ARNT is capable of homodimerizing as well as heterodimerizing with other class I factors such as HIF-1α, SIM1 and SIM2 (Kewley et al. 2004). However, AhR and ARNT are approximately 20% identical in amino acid sequence and show a resemblance in overall structures (Hankinson 1995).

The *AhR* gene is located on human chromosome 7 within p21 and p15 and in the centromeric region of mouse chromosome 12 (Le Beau et al. 1994; Poland et al. 1987;

Schmidt et al., 1993). The *ARNT* gene located on human chromosome 1q21 and mouse chromosome 3 (Johnson et al. 1993).

1.3. AhR complex and transformation by ligand

Prior to activation with ligand, the AhR complex exists as cytoplasmic aggregates and AhR is part of a multimeric protein complex of about 280 kDa (Cuthill et al. 1987) which contains, besides AhR, two 90-kDa heat-shock proteins (HSP90), immunophilin-like proteins including the 43-kDa protein termed hepatitis B virus X-associated protein (XAP2 also known as aryl hydrocarbon receptor interacting protein) and cochaperone p23 (Carver and Bradfield 1997; Ma and Whitlock 1997; Meyer and Perdew 1999; Meyer et al. 1998). HSP90 interacts with the AhR via both the bHLH region and B segment of PAS (Whitlock 1999). HSP90 appears to chaperone a high affinity ligand binding conformation of AhR and is involved in the retention of AhR in the cytoplasm and prevents nuclear translocation, perhaps by masking its nuclear localization sequence present in the amino-terminus of AhR (Pongratz et al. 1992; Whitelaw et al. 1994).

Upon ligand binding, activated AhR is dissociated from HSP90 and translocated to the nucleus in an energy-dependent manner through nuclear localization sequence and nuclear import receptors (Okey et al. 1980; Whitlock 1999). Ligand binding to AhR occurs over the PAS B segment (Ko et al. 1997; Sogawa et al. 1995). In the nucleus, the ligand activated AhR forms a heterodimeric complex with ARNT (Nebert et al. 1993) (Figure 1.1). Both helices of the bHLH region of ARNT and AhR and their PAS domains are required for dimerization (Reisz-Porszasz et al. 1994).

1.4. DNA binding of the AhR/ARNT heterodimer

The AhR/ARNT complex binds to a class of DNA sequences called xenobiotic responsive element (XRE) of the target genes to activate their transcription (Whitlock 1999) (Figure 1.1). The basic regions of both bHLH proteins (AhR and ARNT) and PAS domain are required for DNA binding (Whitlock 1999). In contrast to the classical bHLH transcription factors, which bind the symmetric E-box sequence on DNA, CANNTG, the AhR/ARNT heterodimer binds an asymmetric recognition site, GCGTG sequence within XRE (Swanson and Yang 1999). The carboxy-terminal segments of both AhR and ARNT contain transcriptional activation domains (Ko et al. 1997; Sogawa et al. 1995).

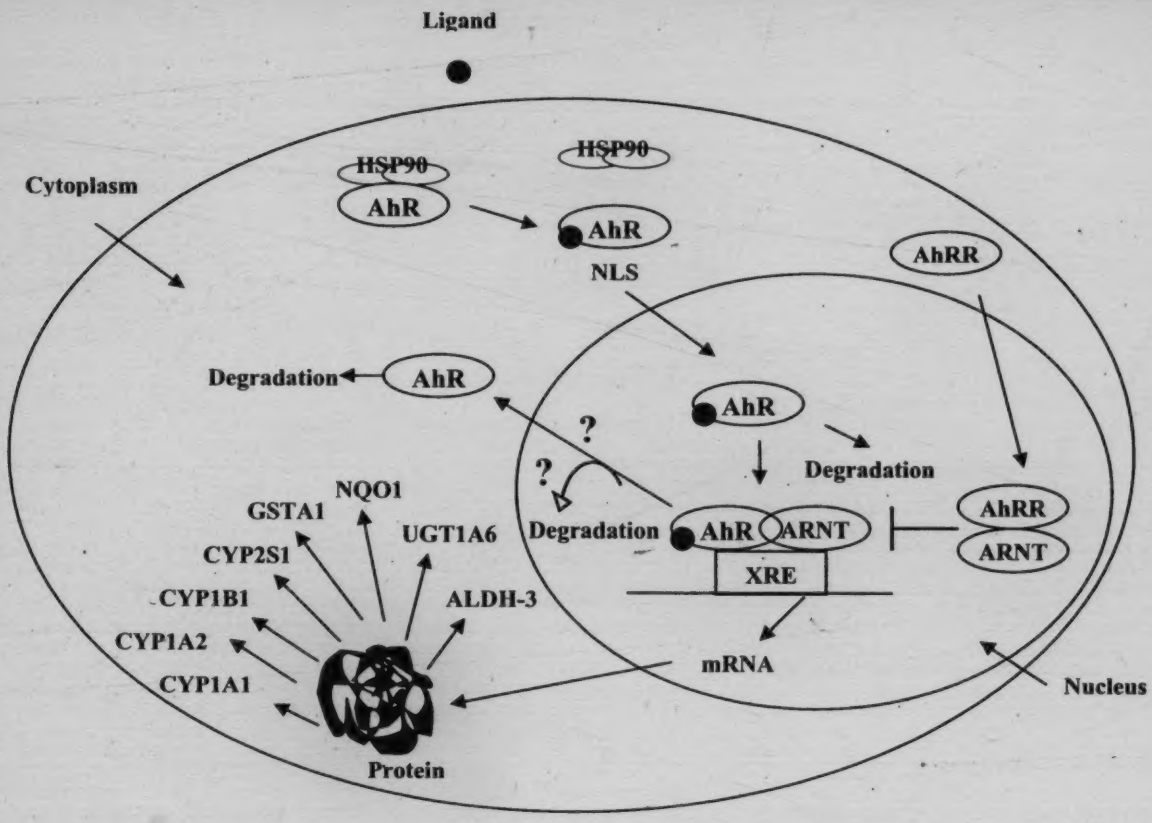

Figure 1.1. A model of the AhR signalling pathway, Ligand binds to the AhR/HSP90/immunophilin complex, causing conformational change and exposing the nuclear localization sequence. Activated AhR is dissociated from HSP90 and translocated to the nucleus. If the ligand/AhR complex is in a misfolded conformation, it may be proteolytically degraded. Activated AhR dimerizes with ARNT. The AhR/ARNT complex binds to XRE regions in DNA. The AhRR/ARNT heterodimer competes with the AhR/ARNT for binding to XRE. The AhR/ARNT complex dissociates from DNA and AhR is ubiquinated in the nucleus and degraded or AhR is exported from nucleus via the chromosome region maintenance protein 1 export receptor. The AhR is ubiquinated in the cytoplasm and targeted to 26S proteasome for degradation.

1.4.1. Role of phosphorylation in DNA binding of the AhR/ARNT heterodimer

Because all of the proteins in the AhR core complex are phosphoproteins, the phosphorylation status of AhR, HSP90, XAP2, or ARNT is likely to modulate AhR transactivation. It has been shown that formation of the AhR/ARNT heterodimer requires only phosphorylation of ARNT (Berghard et al. 1993). However, DNA binding of the AhR/ARNT heterodimer requires phosphorylation of both AhR and ARNT (Mahon and Gasiewicz 1995; Pongratz et al. 1991). Phosphorylation of tyrosine residue(s) plays a critical role in the ability of the AhR/ARNT heterodimer to bind to DNA, while that of serine/threonine residues of either the AhR or ARNT regulates events that occur following DNA binding and steps involved in transcriptional activation (Li and Dougherty 1997; Park et al. 2000).

In addition, several studies have shown that the protein kinase C (PKC) pathway acts as a modifier of serine/threonine residues within the AhR/ARNT proteins, suggesting that activation of the PKC pathway enhances the ability of the AhR/ARNT heterodimer to regulate genes (Berghard et al. 1993; Carrier et al. 1992; Chen and Tukey 1996; Okino et al. 1992). It has been reported that the transactivation domains of AhR and ARNT are not necessary for PKC-mediated AhR/ARNT transactivation (Long and Perdew 1999). However, PKC increases the ability of coactivator proteins to interact with both AhR and ARNT (Long et al. 1999; Long and Perdew 1999). AhR ligands are known to induce PKC activity and the recruitment of c-jun/c-fos, which leads to the activation of other transcription factors such as activator protein-1. Activator protein-1 binds to specific

DNA sequences and in conjugation with the AhR promotes transcriptional activation (Chen and Tukey 1996).

1.4.2. Interaction between AhR and other nuclear proteins

Coregulatory (coactivator and corepressor) proteins have been shown to be required for the regulation of gene expression by many transcription factors including AhR. Coactivators, provide a bridge between AhR molecules, located at 5'-enhancer elements, and general transcription factors, located at the promoter of the gene and physically associate with the gene induction (Hankinson 2005). Consistent with their role in altering chromatin structure, certain coactivators such as steroid receptors-coactivator-1 (SRC-1), CREB-binding protein (CBP) and its structural homologue p300 (p300/CBP), nuclear coactivator-2 (NcoA-2), and p300/CBP cointegrator protein (p/CIP), possessing acetyltransferase activity covalently modify the amino-terminal tails of the core histone proteins in nucleosomes (Hankinson 2005). Several coactivators and corepressors have been shown to interact with the AhR and/or ARNT and modulate the transactivation potential of the heterodimer. Recent studies have shown that in human embryonic kidney cells (293T) and murine hepatoma cells (Hepa-1) AhR directly interact with SRC-1, NCoA-2 and p/CIP, while ARNT directly interacts with SRC-1 and NCoA-2 (Beischlag et al. 2002; Hankinson 2005). Overexpression of SRC-1 family coactivators leads to increased ligand-dependent AhR-reporter activity in Hepa1c1c7 cells (Beischlag et al. 2002). In various cell types, the coactivator RIP-140 is directly bound to AhR, but not to ARNT, which results in enhanced XRE-driven reporter activity (Kumar et al. 1999). Physical and functional interactions of AhR and estrogen receptor with coactivator,

ERAP140, and corepressor, silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), suggest possible competition for limited coregulators, or functional cross-talk through coregulators, which may play a role in cross-talk between AhR and estrogen receptor (Nguyen et al. 1999). It has been shown that the corepressor SMRT directly interacts with AhR. However, because of conflicting findings and the possibility of cell-type-dependent responses, it is not clear whether or not SMRT represses AhR-dependent gene transcription (Rushing and Denison 2002). The relative levels of coactivator versus corepressor proteins play a regulatory role in nuclear receptor cross-talk leading to modulation of gene expression (Swanson 2002).

In the past few years, evidence has emerged to show that the AhR and nuclear factor-κB (NF-κB), a peiotropic transcription factor that is involved in the regulation of inflammatory responses, interact and transcriptionally modulate each other (Tian et al. 2002). Recently it has been demonstrated that the nuclear receptor coactivators CBP, p300/CBP, and SRC-1, and the corepressor, SMRT, also mediate cross-interaction between AhR and NF-κB signalling pathways (Ke et al. 2001). SRC-1, CBP, and SMRT were found to associate with RelA (an active component of NF-κB), AhR, and ARNT (Ke et al. 2001; Kobayashi et al. 2002; Kumar et al. 1999; Lee et al. 2000; Nguyen et al. 1999; Perkins et al. 1997). Comptetition between AhR/ARNT heterodimer and NF-κB for coactivator and corepressor binding could affect the level of transcriptional activation obsereved in these two pathways. Further evidence of cross-talk with NF-κB is supported by the identification of NF-κB binding sites in the 5'-upstream region of an AhR

repressor (AhRR), which are activated by p65/p50 NF-κB heterodimers, resulting in induction of AhRR expression (Baba et al. 2001).

Several studies have reported that AhR-mediated transcriptional activity can influence the cell cycle. Direct interactions between AhR and retinoblastoma protein (Rb) were documented both *in vitro and in vivo*. After AhR transformation and nuclear localization, two domains in AhR directly bind to Rb; however, *in vitro*, ARNT was not required for AhR/Rb interaction (Ge and Elferink 1998; Puga et al. 2000). Interactions of AhR with Rb also appeared to be necessary for maximum TCDD-induced CYP1A1 mRNA expression, suggesting that Rb may act as a coactivator of AhR (Elferink et al. 2001).

There are many studies that supports an interaction between AhR and other nuclear receptors such as, estrogen receptors, androgen receptors, and thyroid receptor pathways (Porterfield 2000; Safe et al. 1998). Estrogen receptor and AhR pathways interact through several different mechanisms that may be cell-type specific. These include: (1) AhR ligands enhance metabolism of 17β-estradiol (Spink et al. 1998). (2) The nuclear AhR complex targets specific genomic core, inhibitory XRE, in promoter regions of some 17β-estradiol-responsive target genes to inhibit hormone-induced transactivation (Safe et al. 1998). (3) AhR ligands induce proteasome-dependent degradation of estrogen receptor-α which is correlated with their binding affinities for AhR (Harris et al. 1990). (4) Treatment with 17β-estradiol resulted in a decrease in CYP1A1 protein levels by blocking AhR-dependent transcription of CYP1A1 due to the

AhR and estrogen receptor-α competition for the transcription factor, nuclear factor-1 (NF-1) (Ricci et al. 1999).

Hypoxia inducible factor (HIF) is another transcription factor that has been shown to interact with the AhR pathway. In response to hypoxia, ARNT is dimerized with HIF-1α. ARNT/HIFα complex binds to hypoxia-responsive elements, leading to transactivation of target genes (Tomita et al. 2000). Under hypoxic conditions in mouse, rat, and human hepatoma cells, AhR-dependent gene expression decreased in the absence of ARNT expression or AhR/ARNT heterodimerization (Pollenz et al. 1999). On the other hand, study in rat hepatoma H4-II-EC3 cells showed that ARNT levels did not affect hypoxia-induced regulation of aldehyde dehydrogenase-3 by the AhR, suggesting that AhR and HIF-1α inhibit each other in a transcription-dependent manner, independent of competition for ARNT (Reisdorph and Lindahl 2001).

1.5. Degradation of AhR

To understand the mechanisms of gene regulation, it is important to investigate the termination phases of inducible transcription. The degradation of AhR would be one of the key factors controlling gene regulation. For example, ligand binding would increase levels of nuclear AhR that would dimerize with ARNT and bind to DNA. However, after the initial accumulation of AhR, its degradation would derive the pathway toward the dissociation of AhR/ARNT complexes and attenuation of the response (Pollenz 2002). Recent reports have suggested that AhR is rapidly down-regulated following ligand binding by degradation (Fujii-Kuriyama and Mimura 2005; Pollenz

2002). Other studies have suggested that the nuclear ligand/AhR complex that fails to dimerize with ARNT and/or bind to DNA must be exported from the nucleus by a nuclear export sequence present in the AhR. Then AhR is ubiquinated and degradated in cytoplasm (Davarinos and Pollenz 1999). In contrast, other studies revealed that AhR was degraded within nuclear compartments through the 26S proteasome pathway (Lees et al. 2003; Roberts and Whitelaw 1999).

Taken together, down-regulation may happen at different steps. Following nuclear translocation of the ligand/AhR complex, if the ligand/AhR complex is in a misfolded conformation, it may be proteolytically degraded. When the AhR/ARNT complex dissociates from DNA, AhR is ubiquinated and degraded in the nucleus or AhR is exported from nucleus via the chromosome region maintenance protein 1 export receptor then is ubiquinated and degraded by 26S proteasome in cytoplasm (Pollenz 2002) (Figure 1.1). Future experiments will need to address whether the AhR and ARNT heterodimer bound to the XRE sequence is degraded by proteasome while bound to the DNA or after release from the XRE sequence.

On the other hand, another cytoplasmic bHLH/PAS transcription factor, AhRR, forms a heterodimer with ARNT. AhRR shows high sequence similarity with the AhR in the N-terminal region up to the end of the PAS A, but does not have ligand binding or a transactivation domain (Mimura et al. 1999). AhRR can form a heterodimer with ARNT to replace the AhR/ARNT complex in association with the XRE sequence and may play a

role in releasing the AhR/ARNT complex from the XRE sequence, facilitating its degradation (Mimura et al. 1999).

1.6. Species distribution of AhR and ARNT

The structural variability of the AhR is significant across species. Photoaffinity labelling of hepatic cytosol indicates that the AhR can vary in molecular weight by almost 30 kilodaltons among different species, e.g. C57 mouse, 95; chicken, 101; guinea pig, 103; rabbit, 104; rat, 106; human, 106; monkey, 113; and hamster, 124 (Poland et al. 1987). Cloning studies have shown that the difference in molecular weight is due to differences in the position of the AhR's translational termination codon, rather than differential splicing or post-translational modification (Carver et al. 1994; Dolwick et al. 1993; Poland et al. 1994; Schmidt et al. 1993).

Cloning of the human and rat AhR cDNAs demonstrated strong N-terminal sequence conservation with the mouse AhR (Carver et al. 1994; Dolwick et al. 1993). The N-terminus of the human AhR shows 100% amino acid identity with the mouse AhR in the basic region, 97% in the HLH, and 87% in the PAS domain. The rat AhR identity with the mouse is 100% in the bHLH and 96% in the PAS domain and with the human AhR is 98% identical in the bHLH domain and 86% in the PAS domain. The C-terminus of AhR among mouse, rat and human are more diverse, however, there is 60% identity between human and mouse, 61% between human and rat, and 79% between rat and mouse (Schmidt and Bradfield 1996).

The primary sequence of the ARNT protein is more conserved between species than the AhR. Cloning of a fragment of rat ARNT corresponding to nucleotides 286-1392 of human ARNT shows 98% overall amino acid identity between the two proteins (Carver et al. 1994; Schmidt and Bradfield 1996). A cDNA-encoding mouse ARNT isolated from Hepa-1 cells shows 92% amino acid identity with human ARNT (Li et al. 1994), although the C-terminal of human and mouse ARNT display greater diversity than the N-terminal (Schmidt and Bradfield 1996).

1.7. Polymorphisms of AhR

Even before the AhR was characterized, it was well-established that various strains of inbred mice differ in their responsiveness to AHH induction by TCDD (Harper et al. 2002). Studies with recombinant inbred mice showed polymirphisms within the coding region of AhR of various inbred strains of mice. The highest susceptibility to toxic effects of TCDD and the highest sensitivity to CYP1A1 induction occurs in the "responsive" strain, C57BL/6 mice with the high affinity Ahr^{b1} allele (Nebert 1989; Poland and Glover 1990). The "non-responsive" strain, DBA/2 mice, is less sensitive than C57BL/6 to biochemical and toxic effect of TCDD including lethality, teratogenicity, hepatic porphyria and thymic atrophy and is homozygous for the low affinity Ahr^{d} allele. The affinity of AhR for TCDD is about 10-fold higher in mice with the Ahr^{b1} allele than in mice with the Ahr^{d} allele (Okey et al. 1989). For instance, hepatic lipid peroxidation induced by TCDD occurred at low doses (0.5 μg/kg) in C57BL/6 mice and only at higher doses (5 μg/kg) in DBA/2 mice (Mohammadpour et al. 1988). The decreased affinity of the Ahr^{d} allele for TCDD is due to a single nucleotide change at

codon 375 that causes valine to be substituted for alanine in the ligand-binding domain of the AhR (Ema et al. 1994; Poland et al. 1994). Recent study on sequence variations in the *AhR* gene from a large number of *Mus musculus* strains and subspecies has shown the existence of an average of 14 variants per kilobase in exons and 20 variants per kilobase in intronic regions. These genetic variations predict 42 different amino acid changes in the mouse AhR protein (Thomas et al. 2002).

In rats, in the sensitive Long-Evans strain, TCDD-incduced toxicity is 1000-fold greater than that in the resistant Han/Wistar strain (Pohjanvirta and Tuomisto 1994). The disparity in sensitivity to TCDD between Han/Wistar strain and other strains can be attributed to variation in the primary structure of the AhR (Pohjanvirta et al. 1998; Tuomisto et al. 1999). The *AhR* gene in the Han/Wistar strain carries a point mutation, which is not in a coding region but rather is in the first intronic nucleotide lying on the 3′-side of exon 10. Exon 10 encodes the major AhR region that is implicated in transactivation of AhR-target genes, particularly *CYP1A1* (Jain et al. 1994; Ma et al. 1995; Whitelaw et al. 1994). The intronic mutation creates splice variants that delete either 38 or 43 amino acids from the transactivation domain of the AhR in Han/Wistar strain (Pohjanvirta et al. 1998).

In human, the most studied polymorphism of AhR involves the substitution of arginine to lysine at codon 554 (Harper et al. 2002). Interpretation of the phenotypic effects of the *AhR* polymorphism at codon 554 remains controversial. Studies on Japanese or French subjects showed no significant effect of the AhR polymorphism at

codon 554 on AHH or EROD induction in cultured lymphocytes, respectively (Cauchi et al. 2001; Kawajiri et al. 1995). In addition, the polymorphism at codon 554 was not associated with neither EROD activities nor CYP1A1 mRNA or protein levels in lung tissues of smokers (Anttila et al. 2000; Smith et al. 2001). However, in Caucasian subjects EROD activities was higher in lymphocytes from heterozygous Arg554/Lys554 or homozygous Lys554/Lys554 individuals than in homozygous Arg554/Arg554 individuals (Smart and Daly 2000). Moreover, polymorphisms at codons 517 and 570 have been shown to have a strong effect on the phenotype of an AhR-mediated response (Harper et al. 2002). However, more human AhR polymorphisms certainly remain to be discovered.

1.8. Tissue distribution of AhR and ARNT

AhR complex ligand binding activity, AhR protein, AhR mRNA, ARNT mRNA, and CYP1A1 induction by AhR ligands occurred in most examined rodent and human tissues, although the degree of expression of each varies considerably between different tissues (Hankinson 1995).

Northern blot analysis of RNA from eight different human tissues showed that the human AhR is highly expressed in placenta, lung, liver, heart, and pancreas, and with lower levels of expression found in kidney, brain, and skeletal muscle. On the other hand, ARNT is expressed in the liver, placenta, and chorion (Brooks et al. 1989; Dolwick et al. 1993). Ribonuclease protection assays of rat tissues showed that the rat AhR was highest in lung, thymus, liver, and kidney and lowest in heart and spleen (Carver et al. 1994). In

contrast to the human placenta, AhR mRNA is not highly expressed in rat placenta. This finding may reflect a species difference in AhR expression or may result from different gestational ages of the two tissue samples. The rat ARNT mRNA was also ubiquitously expressed and the result of ribonuclease protection assays showed that AhR was highest in placenta, lung, and thymus and lowest in spleen, brain, and heart (Carver et al. 1994). In general, the AhR and ARNT proteins appear to be co-expressed; however, differences in relative expression levels exist between the two proteins in some tissues. Tissues in which one protein is present in excess over the other may indicate the existence of additional dimerization partners and signalling pathways. In addition, low levels of ARNT could decrease the sensitivity of a particular tissue to ligand despite high AhR levels (Schmidt and Bradfield 1996).

The AhR and ARNT were found to be present at different levels in many embryonic tissues. Both the AhR and ARNT are already expressed at gestational day 10–11, with the highest levels in heart, neuroepithelium and neuroepithelial/neural crest-derived tissues. The AhR is also expressed in facial membranous bone. At gestational day 12–13, brain and heart levels of AhR and ARNT have decreased, and the highest levels are now found in the liver. Areas of bone formation, epithelium of gut, lung, and kidney retain high levels of the AhR. ARNT is also expressed at significant levels in the tongue. At gestational day 14–16, both the AhR and ARNT remain strongly expressed in the liver and are also high in adrenal gland and developing bone. AhR is also expressed in the epidermis (Abbott et al. 1995; Abbott and Probst 1995).

1.9. AhR ligands

AhR ligands have been separated into two major categories, those that are formed as a result of non-biological activity and those that formed in biological systems as a result of natural process. To date, numerous chemicals have been identified as AhR ligands (Denison et al. 2002). Most of them, the "classical" ligands, including polycyclic aromatic hydrocarbons (PAHs) (such as 3-methlycholantherene (3-MC), benzo[a]pyrene, and β-naphthoflavone) and halogenated aryl hydrocarbons (HAHs) (such as polyhalogenated dibenzo-*p*-dioxins, dibenzofurans, and biphenyl), share the structural features of being planar, aromatic, and hydrophobic (Denison and Nagy 2003). The most potent class of AhR ligands are the metabolically more stable HAHs, with binding affinities in the pM to nM range, whereas the metabolically more labile PAHs bind with relatively lower affinity (nM to μM range). Structure-activity relationship analysis using a large number of HAHs and PAHs has suggested that the AhR ligand binding pocket can bind planer ligands with maximal dimensions of 14 A^0 x 12 A^0 x 5A^0 and that high affinity ligand binding appears to be dependent upon key electronic and thermodynamic characteristics of the ligand (Kafafi et al. 1993; Mhin et al. 2002; Tuppurainen and Ruuskanen 2000; Waller and McKinney 1995). A wide range of structural diversity in AhR ligands indicates that a greater spectrum of chemicals can interact with and activate AhR than previously thought (Denison and Nagy 2003). Recently, a relatively large number of AhR ligands whose structures and physiochemical characteristics differ from classical ligands have been identified. The majority of these "non-classical" AhR ligands have a low affinity for the AhR and are relatively weak inducers of CYP1A1, compared to TCDD (Denison and Nagy 2003).

1.9.1. Natural dietary AhR ligands

Diet is the greatest source of exposure of animals and humans to AhR ligands. Several studies have described and characterized a variety of naturally occurring dietary chemicals that can directly activate and/or inhibit the AhR pathway (Denison and Nagy 2003). Some of these chemicals are widely distributed in dietary vegetables, fruits, and teas (Formica and Regelson 1995). Several studies have reported that extracts of vegetables or vegetable-derived materials could induce CYP1A1 activity (Bjeldanes et al. 1991; Wattenberg and Loub 1978). In addition, the ability of several dietary plant compounds such as indole-3-carbinol, 7,8-dihydrorutacarpine, dibenzoylmethanes, curcumin, β-apo-8-carotenal, cartinoids, and apo-carotinoid to competitively bind to the AhR and/or induce AhR-dependent gene expression was reported (Bjeldanes et al. 1991; Ciolino et al. 1998a; Gillner et al. 1993; Gillner et al. 1989; Gradelet et al. 1996a; Gradelet et al. 1996b; MacDonald et al. 2001). Some studies reported that the dietary indoles in the mammalian digestive tract are significantly more potent AhR ligands (Bjeldanes et al. 1991; Perdew and Babbs 1991). In fact, indole [3,2-b]carbazole, an acidic condensation product formed from indole-3-carbinol which is a weak ligand, may have the highest affinity of any "natural" AhR ligand identified to date (~0.2-3.6 nM). It is also a potent inducer of AhR-dependent gene expression in cell cultures (Bjeldanes et al. 1991; Gillner et al. 1993).

Among flavonoids, numerous AhR agonists such as quercetin, diosmin, tangeritin, and tamarixetin have been identified. However, the majority of these natural plant products such as flavone derivatives are AhR antagonists (Allen et al. 2001; Ashida

2000; Ashida et al. 2000; Canivenc-Lavier et al. 1996; Ciolino et al. 1999; Ciolino et al. 1998b; Henry and Gasiewicz 2003; Obermeier et al. 1995; Yannai et al. 1998). They bind to the AhR but are unable to elicit the XRE-binding conformation of the receptor (Gasiewicz et al. 1996; Henry and Gasiewicz 2003; Mahon and Gasiewicz 1992). Therefore, ligand binding alone is insufficient to initiate the necessary steps leading to receptor transformation. Agonist ligands initiate structural alteration in the AhR that is ARNT-dependent and at least partially involves the ligand-binding /PAS domain (Henry and Gasiewicz 2003).

1.9.2. Endogenous AhR ligands

Several studies have suggested the existence of endogenous physiological AhR ligands, which activate the AhR signalling pathway in the absence of exogenous ligands. The demonstration that disruption of AhR expression using antisense resulted in decreased development of mouse blastocytes and alterations in normal cell cycle progression combined with the identification of nuclear AhR complexes in untreated cell cultures and tissue slices support the existence of endogenous AhR ligands (Abbott et al. 1994; Chang and Puga 1998; Ma and Whitlock 1996; Singh et al. 1996; Weiss et al. 1996). The occurrence of several physiological changes and developmental abnormalities in AhR knockout animals provides the best evidence for a role of the AhR in normal development and physiological/biochemical processes (Desmots et al. 2001; Lahvis et al. 2000; Schmidt and Bradfield 1996). These changes may result from loss of AhR activation by an endogenous ligand, although the identity of the responsible chemical(s) remains to be determined (Denison and Nagy 2003).

Recently, many endogenous compounds such as tryptamine, indole-acetic acid (Heath-Pagliuso et al. 1998), bilirubin, biliverdin (Phelan et al. 1998; Sinal and Bend 1997), and lipoxin A4 (Schaldach et al. 1999) have been isolated as potential natural ligands of the AhR. These compounds have relatively low binding affinities for the AhR in comparison to those of TCDD, 2,3,7,8-tetrachlorodibenzofuran. However, the tryptophan-derived natural AhR ligand, 6-formylindolo[3,2-b]carbazole, has a very high affinity for AhR comparable to that of TCDD (Wei et al. 1998).

Based on the structural diversity and general hydrophobic nature of AhR ligands, it seems reasonable to suggest that some biological lipids and/or steroids may be endogenous AhR ligands (Denison and Nagy 2003). To support this hypothesis, some studies have shown that lipoxin A4, a peroxygenase product of arachidonic acid (AA), and prostaglandins G2 bind to AhR and activate AhR-dependent gene expression (Schaldach et al. 1999; Seidel et al. 2001). Lipoxin A4 induces a transient expression of CYP1A1 at concentrations near physiological levels in some situations (Serhan and Sheppard 1990) and is known as a competitive substrate for CYP1A1 (Schaldach et al. 1999). However, the prostaglandins induce AhR-dependent gene expression only at concentrations higher than 1 μM which is much greater than their normal physiological levels (Seidel et al. 2001; Smith et al. 2001).

Although these chemicals have the potential to activate AhR activity, identification of a true physiological ligand for AhR would require clarification of how

the activation of the AhR by these naturally occurring ligands is associated with specific physiological functions (Denison and Nagy 2003).

1.10. Ligand-independent activation of AhR

Interestingly, some chemicals, including omeprazol, caffeine, nicotine, and primaquine, have been identified that can induce AhR-dependent gene expression without competitively binding to the AhR (Daujat et al. 1992; Fontaine et al. 1999; Galtier et al. 1985; Goasduff et al. 1996; Iba et al. 1998). It has been proposed that these chemicals are not AhR ligands themselves, but they can indirectly activate AhR-dependent gene expression, either through metabolic activation into a ligand or by their ability to affect some signal transduction pathway such as phosphorylation that results in AhR activation (Denison and Nagy 2003). It has been suggested that the nuclear accumulation of AhR is regulated by the phosphorylation of AhR serine residue. Use of specific kinase inhibitors has suggested that this phosphorylation is catalyzed by p38 mitogen-activated kinases (MAPK) (Ikuta et al. 2004). Several studies have reported that phosphorylation regulates AhR activity in the physiological signalling pathway as well as the xenobiotic signal transduction pathway (Puga et al. 2002). Omeprazole induces CYP1A1 in an AhR-dependent manner without binding directly to the AhR (Dzeletovic et al. 1997). Tyrosine kinase inhibitors, tyrphostins AG17 and AG879, selectively inhibited omeprazole-mediated AhR signalling, but did not affect TCDD-mediated induction of CYP1A1. Mutational analysis provided evidence that a Tyr320Phe mutation abolished omeprazole-dependent AhR activation, while the TCDD-dependent activation of CYP1A1 transcription was only minimally affected. These results suggest that Tyr320

is a putative phosphorylation site on the AhR activated by omeprazole in a ligand-independent manner through a signal transduction pathway that involves protein tyrosine kinases. This pathway is independent from that induced by TCDD (Backlund and Ingelman-Sundberg 2005).

2. AHR-REGULATED XENOBIOTIC METABOLIZING ENZYMES

2.1. Introduction

Xenobiotic metabolizing enzymes (XMEs) play important roles in the metabolism, detoxification, and/or elimination of exogenous compounds introduced into the body as well as certain endogenous compounds (Meyer 1996). In order to minimize the potential injury caused by these compounds, most of the tissues and organs are well equipped with diverse and various XMEs, including phase I and phase II metabolizing enzymes, which are present in abundance either at the basal level and/or inducible level after exposure to xenobiotics (Meyer 1996; Rushmore and Kong 2002).

Phase I reactions functionalizes the xenobiotics by introducing or uncovering a chemically reactive functional group to form reactive metabolites through oxidative reactions such as *N*-dealkylation, *O*-dealkylation, hydroxylation, *N*-oxidation, *S*-oxidation or deamination (Gibson and Skett 2001). Phase I XMEs consist primarily of the cytochrome P450 (CYP) superfamily. The reactions of phase I prepare the xenobiotics for phase II reactions (Gibson and Skett 2001). In general, conjugation with phase II XMEs makes a pharmacologically inactive compound and/or increases hydrophilicity, and enhances excretion in the bile and/or the urine and consequently detoxifies the harmful xenobiotics (Hinson and Forkert 1995). Phase II XMEs consist of many superfamily of enzymes including, glutathione S-transferases (GST), UDP-glucuronosyltransferases, sulfotransferases (UGT), NAD(P)H:quinone oxidoreductase 1 (NQO1), expoxide hydrolases, aldehyde dehydrogenase (ALDH), and *N*-

acetylteransferases (NAT) (Xu et al. 2005). Each superfamily of phase I and II XMEs consists of families and subfamilies that are classified based on their amino acid sequence identities (Xu et al. 2005). Many of the reactions of both phase I and II XMEs are capable of being performed on the same compound and there is possibility of interaction of the various metabolic routes in terms of competing for the same substrate (Gibson and Skett 2001). Phase I and II XMEs are regulated through different mechanisms. In the following sections we are discussing about those enzymes, which are regulated via AhR pathway.

2.2. Cytochrome P450

The CYPs contain red-pigmented heme, which absorb light at a wavelength of 450 nm following bound to carbon monoxide. In the term *cytochrome P450*, *cyto* stands for cell, *P* for *pigments* and *450* for *450 nm* (Omura and Sato 1964). The ability of reduced CYP to induce an absorption peak at 450 nm upon carbon monoxide binding is currently used to estimate CYP content (Omura and Sato 1964). These heme-containing proteins are closely associated with nicotinamide adenine dinucleotide phosphate (reduced formed) (NADPH) cytochrome P450 reductase, which was first shown to participate in the oxidation of many xenobiotics and associated with the microsomal fraction of liver (La Du 1955). CYPs are located in the membrane of smooth endoplasmic reticulum or in some cases in the inner membrane of mitochondrial and are found abundantly in the liver, gastrointestinal tract, lung, kidney, heart, and brain (Elbekai and El-Kadi 2006; Gonzalez and Nebert 1990; Guengerich 2003; Meyer 1996; Nebert et al. 1991; Nelson et al. 1996; Oinonen and Lindros 1998; Renton and Nicholson 2000).

Ever since initial purification and isolation of CYP in a catalytically component form in the late 1960s, many studies have emphasised that CYP exists as multiple forms derived from separate and distinct genes. To date, around 4000 different isoenzymes of CYP have been identified (Nelson 2006). The nomenclature system for CYP, first devised in 1987, groups enzymes and genes into families and subfamilies with the prefix CYP used to designate the CYP enzymes in all species (except mouse gene, where Cyp is used). This system relies on evolutionary relationship as depicted in phylogenic trees (Nelson 2006). In this system, families are designed by an Arabic number, with all numbers of a particular family having more than 40% identity in their amino acid sequence. A subfamily consists of enzymes in which the amino acid sequence is more than 55% identical. The nomenclature for CYP used to be small, now due to great diversity of CYP in some species like insects, fungi and bacteria there is need for additional levels of nomenclature, above the family and superfamily. The concept of clans has been proposed as a level above family rank (Nelson 2006).

CYPs, which catalyze mono-oxygentation reactions, are important in the biosynthesis and degradation of endogenous compounds such as steroids, lipids, and vitamins. They metabolize and alter the pharmacological activity of many chemicals present in the diet and environment, as well as drugs (Oinonen and Lindros 1998). In human, the 5 CYP gene families CYP1, CYP2, CYP3, CYP4 and CYP7 are believed to play crucial roles in hepatic as well as extra-hepatic metabolism and elimination of xenobiotics and drugs (Gonzalez and Nebert 1990; Nebert et al. 1991; Nelson et al. 1996; Pascussi et al. 2003; Simpson 1997; Waxman 1999).

2.3. AhR-regulated phase I xenobiotic metabolizing enzymes

Much of the understanding of the AhR pathway comes from analysis of the regulatory regions controlling expression of the genes encoding the phase I XMEs such as cytochrome P450 1A1 (CYP1A1), 1A2, and 1B1 that contribute to AHH activity (Jones et al. 1986; Quattrochi et al. 1994; Schmidt and Bradfield 1996). Analysis of the 5' regulatory regions of the *CYP1A1* gene demonstrated the existence of XREs that were bound to the AhR/ARNT heterodimer and were required for enzyme induction (Denison et al. 1988a; Denison et al. 1988b; Fujisawa-Sehara et al. 1988; Schmidt and Bradfield 1996). Deletion analysis of the human *CYP1A2* promoter has also revealed that XRE regions are required for TCDD inducibility of this gene (Quattrochi et al. 1994). Moreover, *CYP1B1* and *CYP2S1* have been discovered to be TCDD-inducible *CYP* genes, and their promoter also appears to be regulated by a similar mechanism (Rivera et al. 2002; Schmidt and Bradfield 1996; Spink et al. 1998) (Figure 1.1).

2.3.1. CYP1A subfamily

The CYP1A subfamily contains two highly homologous and well-characterized but distinct members; CYP1A1 and CYP1A2 (Toussaint et al. 1993). They are found in fungi, plant, insect, different animals such as mouse, rat, rabbit, dog, cattle, and human (Guengerich et al. 1982; Nelson et al. 1996). In mice, CYP1A1 mRNA has been detected at very low levels in liver, lung, heart, kidney, intestine, thymus, testis, uterus, ovary, and brain which is highly induced in these organs by AhR ligands (Shimada et al. 2003). In rats and humans, CYP1A1 is expressed at low level in liver, kidney, lung, intestine, brain, placenta, and heart (Chinta et al. 2005; Czekaj et al. 2005; Guengerich 2003; Omiecinski

et al. 1990). Among the AhR-regulated genes, CYP1A1 is the most capable of producing polar, toxic or even carcinogenic metabolites from various AhR ligands, including aromatic and halogenated hydrocarbons (Schrenk 1998). These metabolites have been shown to be involved in the mediation of a broad range of distinct toxic responses such as immune suppression, endocrine disruption, birth defects, and carcinogenesis (Poland and Knutson 1982). CYP1A1 capacity to activate tobacco-smoke-derived PAHs is the putative role of this CYP form in lung carcinogenesis (Guengerich 1993). The *CYP1A1* gene is also induced by endogenous compounds such as AA metabolites, bilirubin, biliverdin, indigo, and indirubin (Denison and Nagy 2003; Guengerich et al. 1982; Sinal and Bend 1997). Some studies have reported that negative regulatory elements and cognate repressor proteins in promoter regions of *CYP1A1* negatively modulate the expression of this gene (Boucher et al. 1995; Boucher et al. 1993). In rat and human cells, mutations in the cognate repressor protein inhibited DNA/protein binding, resulting in a two- to three-fold increase in the maximal CYP1A1 inducibility in response to AhR ligand, suggesting the presence of a negative regulatory elements in the 5' region of the *CYP1A1* promoter gene (Boucher et al. 1995; Jorgensen and Autrup 1995; Piechocki and Hines 1998; Walsh et al. 1996). These observations was further supported by the studies on murine hepatoma Hepa 1c1c7 and human breast epithelial MCF10A cells which showed co-treated the cells with a protein synthesis inhibitor, cycloheximide, and TCDD superinduced CYP1A1 mRNA level (Joiakim et al. 2004; Korashy and El-Kadi 2006).

The *CYP1A2* gene is constitutively expressed in hepatic and extrahepatic tissues such as heart and brain (Goldstein et al. 1977; Kapoor et al. 2006; Minamiyama et al.

1999). It was suggested that induction of CYP1A2 is restricted to the liver whereas CYP1A1 is inducible in both hepatic and many extrahepatic tissues (Dey et al. 1999). In addition to its inducibility by PAHs and aromatic hydrocarbons, CYP1A2 also play a role in the bioactivation of xenobiotics, such as PAH, aromatic amines, and heterocyclic amines (Quattrochi et al. 1998). Both AhR-specific and tumor promoter-specific elements regulate the expression of human *CYP1A2* gene, suggesting the involvement of AhR as well as AhR-independent mechanisms in CYP1A2 induction (Quattrochi et al. 1998).

The human CYP1A2, along with other CYPs, catalyzes the metabolism of many xenobiotics and drugs, including caffeine, theobromine, theophylline, verapamil, lidocaine, propafenone, clozapine, tacrine, chlorzoxazone and dantrolene as well as the bioactivation of procarcinogens, including PAHs, nitrosamines and arylacetamides (MacDonald et al. 2001; Spatzenegger and Jaeger 1995). CYP1A2 also catalyzes the oxidation of aromatic amines and 17β-estradiol and may also be important in 4-hydroxylation of tamoxifen (Kupfer et al. 1994).

2.3.2. CYP1B1 subfamily

CYP1B1 is expressed in humans at a low level in many tissues other than liver (Spink et al. 1998). There is still controversy regarding CYP1B1 expression in lung tissue (Hukkanen et al. 2002). Among the different CYP isoforms, overexpression of CYP1B1 is the one most often detected in various tumors (Murray 2000). Human CYP1B1 has been shown to express in breast tumors and metabolize 17β-estradiol to a 4-hydroxylated product, a chemical considered to cause breast cancer in women (Spink et al. 1998).

CYP1B1 is also constitutively expressed in rat and mouse adrenal and ovary. It is induced in several organs, including liver, by TCDD and other PAHs (Bhattacharyya et al. 1995; Shen et al. 1994; Shimada et al. 2003) in both rodents and humans. CYP1B1 participates in the metabolic activation of a number of procarcinogens, including benzo[a]pyrene and arylamines, at rates similar to or even higher than CYP1A1 in experimental animals and humans (Hukkanen et al. 2002). It is known that *CYP1B1* gene is transcriptionally activated by PAHs or HAHs through the AhR/ARNT heterodiemr. However, there is evidence that the constitutive Cyp1b1 mRNA and protein were expressed in ARNT-deficient murine hepatoma cells at levels similar to those seen in TCDD-induced wilde-type cells (Eltom et al. 1999). In addition, studies on the human cancer cell lines showed that CYP1B1 protein level was expressed only after treatment with 3MC, while CYP1B1 mRNA levels were equivalent in both the treated and untreated cell lines (McFadyen et al. 2003). These results suggest that a post-transcriptional and AhR-independent mechanisms also contributed the regulation of CYP1B1 (McFadyen et al. 2003).

2.3.3. CYP2S1 subfamily

CYP2S1 is a novel CYP which exhibits TCDD-inducibility mediated by AhR (Rivera et al. 2002). In both human and mouse, CYP2S1 appears to be principally expressed in epithelial cells of a wide variety of extrahepatic tissues more than liver. The highest expression of CYP2S1 has been observed in skin and the epithelial tissues frequently exposed to xenobiotics such as the respiratory, gastrointestinal, and urinary tracts (Rylander et al. 2001; Saarikoski et al. 2005). The ubiquitous tissue distribution, as

well as the expression of CYP2S1 throughout embryogenesis, suggests that CYP2S1 is likely to metabolize endogenous substrates (Du et al. 2004; Smith et al. 2003). CYP2S1 has been shown to be inducible by coal tar, an abundant source of PAHs (Smith et al. 2003). A recent report indicated that CYP2S1 metabolized naphthalene, suggesting the involvement of this CYP isoform in the metabolism of toxic and carcinogenic compounds, similar to other TCDD-inducible CYPs (Karlgren et al. 2005).

2.4. AhR-regulated phase II xenobiotic metabolizing enzymes

Four phase II XMEs, including NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferase A1 (GSTA1), cytosolic aldehyde dehydrogenase-3 (ALDH-3) and UDP-glucuronosyltransferase 1A6 (UGT1A6) are also induced by PAHs and HAHs through AhR-dependent pathway (Asman et al. 1993; Favreau and Pickett 1991; Jaiswal 1994; Paulson et al. 1990) (Figure 1.1). Emerging evidence has demonstrated that NQO1, GSTA1 and UGT1A6 are modulated through both AhR-mediated and non-AhR-mediated pathways. It has been shown that a transcription factor, nuclear factor erythroid 2-related factor-2 (Nrf2) is also involved in the regulation of *NQO1, GSTA1* and *UGT1A6* gene expressions against oxidative damage. Some studies have reported that there is a cross-talk between AhR and Nrf2 signalling pathways (Noda et al. 2003; Zhu et al. 2005). It has been shown that inducible expression of *NQO1* by TCDD not only is controlled by CYP1A1 activity but also requires Nrf2. In addition, AhR activation directly contributes to the regulation of *Nrf2* gene expression (Assenat et al. 2004; Marchand et al. 2004). UGT1A6 is also controlled by other transcription factors including pregnane X receptor and constitutive androstane receptor (Bock and kohle 2005).

2.4.1. NAD(P)H:quinone oxidoreductase 1 (NQO1)

NAD(P)H:quinone oxidoreductase 1 (NQO1) is a homodimeric flavoprotein that has been expressed in various species, including mouse, rat, human, and zebrafish (Jaiswal 2004; Kobayashi et al. 2002; Nebert et al. 1993; Williams et al. 1999). NQO1 is an important enzyme involved in defence against reactive oxygen species (Nioi and Hayes 2004). Vehicle exhaust and cigarette smoke contain compounds such as benzo[a]pyrene that, following cellular metabolism by CYP, yield quinine. Endogenous catechol quinones, which are derived from estrogen, as well as exogenous quinines are highly reactive molecules that can initiate cancer and neurodegenerative diseases (Cavalieri et al. 2004; Nioi and Hayes 2004). Fully oxidized quinones can undergo single-electron reduction reactions and be catalyzed by cytochrome P450 reductase. This reaction results in production of reactive semiquinone intermediates, which can directly form adducts with cellular macromolecules including DNA. They can also undergo redox-cycling to generate further reactive species that are carcinogenic (Joseph and Jaiswal 1994; Nioi and Hayes 2004). Under oxidative stress, NQO1 expression is induced and provides the cell with at least three defensive strategies. Firstly, the catalytic activity of NQO1 is directed towards the complete reduction and detoxication of highly reactive quinones. Secondly, NQO1 maintains the endogenous lipid-soluble antioxidants, alpha-tocopherol-hydroquinone and ubiquinol, in their active forms. Thirdly, NQO1 is required for the stabilisation of p53 transcription factor, a tumour suppression protein, in response to DNA-damaging stimuli, and it influences cell survival (Nioi and Hayes 2004).

Recent immunohistochemical studies in humans have shown NQO1 protein is expressed in many tissues, including the epithelial cells of lung, breast, and colon, vascular endothelium, adipocytes, corneal and lens epithelium, retinal pigmented epithelium, optic nerve and nerve fibers (Khatsenko et al. 1997). It has been also reported that in rats, NQO1 is expressed in liver, kidney, lung, brain, heart, and bladder (Gustafson et al. 2003). In mice, NQO1 is expressed in liver and heart whereas brain, spleen, lung, and skeletal muscle showed undetectable levels of NQO1 gene expression (Muntane et al. 1995).

2.3.2. Glutathione S-transferase (GST)

GSTs catalyze nucleophilic attack by reduced glutathione on compounds that contain an electrophilic carbon, nitrogen, or sulphur atom and play an important role in removing the toxic electrophilic compounds (Hayes et al. 2005). Three major families of GST are cytosolic and mitochondrial which comprise soluble enzymes and microsomal which is membrane associated protein (Jakobsson et al. 1999; Ladner et al. 2004; Robinson et al. 2004). Besides catalyzing conjugation and reduction reactions, cytosolic GST also bind, covalently and noncovalently, to hydrophobic nonsubstrate ligands (Hayes and Pulford 1995). This type of activity contributes to intracellular transport, sequestration, and disposition of xenobiotics and hormones including azo dyes, bilirubin, heme, PAHs, steroids, and thyroid hormones (Hayes and Pulford 1995). Based on amino acid sequence similarities, seven classes of cytosolic GST including Alpha, Mu, Pi, Sigma, Theta, Omega, and Zeta are recognized in mammalian species (Armstrong 1997; Hayes and McLellan 1999; Hayes and Pulford 1995; Sheehan et al. 2001). Other classes

of cytosolic GST, namely Beta, Delta, Epsilon, Lambda, Phi, Tau, and the "U" class, have been identified in nonmammalian species (Ding et al. 2003; Sheehan et al. 2001). In rodents and humans, cytosolic GST isoenzymes within a class typically share >40% identity, and those between classes share <25% identity. In human, at least 16 cytosolic GST subunits exist (Hayes et al. 2005; Hayes and Pulford 1995). Class Alpha GST has four subfamilies including GSTA1, GSTA2, GSTA3, and GSTA4. In human, GSTA4 is expressed in many tissues, including liver, kidney, skin, muscle, and brain (Desmots et al. 2001). However, GSTA1 and GSTA2 are expressed at high levels in liver, intestine, kidney, adrenal gland, pancreas, and testis (Coles et al. 2000; Coles et al. 2002; Coles et al. 2001; Hayes and Pulford 1995; Morel et al. 2002; Mulder et al. 1999; Rowe et al. 1997); and GSTA3 is expressed in steroidogenic tissues and adrenal gland (Johansson and Mannervik 2001). In mice, Gsta1/2 were mainly expressed in liver, kidney and lung (Ruiz-Laguna et al. 2005).

2.3.3. UDP-glucuronosyltransferase (UGT)

UGTs are a family of membrane-bound enzymes on the luminal surface of the endoplasmic reticulum (Dutton 1975). Glucuronidation represents an important pathway which enhances the elimination of many lipophilic xenobiotics and endobiotics to more water-soluble compounds (Zhou et al. 2005). Based on their capacity to catalyze the glucuronidation of xenobiotics and endobiotics, UGTs play a central role in hormonal homeostasis, energy metabolism, bilirubin clearance, and xenobiotic detoxification (Jansen et al. 1995; Owens and Ritter 1995; Qatanani et al. 2005; Xie et al. 2003; Zhou et al. 2005).

Based on the similarities of the DNA and amino acid sequences, the human UGT superfamily is comprised of 2 families (UGT1 and UGT2) and 3 subfamilies (UGT1A, UGT2A, and UGT2B) (Court et al. 2001). Members of the UGT1 family share more than 50% identity with each other but less than 50% identity with members of the UGT2 family (Court et al. 2001). Many of the individual UGT enzymes are expressed not only in liver but also in extrahepatic tissues such as kidney, intestine, brain, and placenta (Collier et al. 2004; Fisher et al. 2001; King et al. 1999; Strassburg et al. 1997). Among UGT isoenzymes, UGT1A6 was the first member of the rat and human UGT1 family was discovered. It is expressed in liver and extrahepatic tissues such as intestine, kidney, testis, and brain (Bock and Kohle 2005). Serotonin has been identified as a selective endogenous substrate of the human UGT1A6 enzyme (Krishnaswamy et al. 2003). UGT1A6 is involved in conjugation of planar phenols, arylamines and drugs such as acetaminophen and phenolic metabolites of benzo[a]pyrene (together with rat UGT1A7 and human UGT1A9) (Bock and Kohle 2005; Court et al. 2001).

2.3.4. Aldehyde dehydrogenase (ALDH)

The ALDHs represent a superfamily of NADP-dependent enzymes having similar primary structures and which eliminate a wide spectrum of toxic endogenous and exogenous aliphatic and aromatic aldehydes (Harrington et al. 1987; Jakoby and Ziegler 1990; Mitchell and Petersen 1987). Twelve aldehyde dehydrogenase (ALDH) genes have been identified in humans. Some of them, including ALDH-1, ALDH-3, and ALDH-9, are cytosolic and some are mitochondrial enzymes. ALDHs are expressed in both hepatic and extrahepatic tissues such as brain and red blood cells. ALDH-3 is strongly expressed

in the stomach and lung, but at a low level in the normal liver and is regulated by AhR signalling pathway (Ambroziak and Pietruszko 1993; Shibuya et al. 1994; Yoshida 1992). ALDH-3 is induced by PAHs and is strongly expressed in about 70% of poorly differentiated and 30% of well differentiated human hepatocellular carcinomas (Shibuya et al. 1994).

3. REGULATION OF XENOBIOTIC METABOLISM ENZYMES DURING INFLAMMATION

3.1. Inflammation

Inflammation is a physiological response to a variety of stimuli such as infections, tissue damage, burns, trauma, tumors, and autoimmune disease and which is characterized by redness, heat, swelling, and pain. Infection or tissue injury induces complex cascades of non-specific events, known as the inflammatory response, which provide early protection by restricting tissue damage to the site of infection or tissue injury. Inflammation involves immune-system cells and numerous mediators (Goldsby et al. 2003). The acute inflammatory response involves both localized and systemic responses. In some diseases persistent immune activation can result in chronic inflammation, which often has pathologic consequences (Goldsby et al. 2003).

Cytokines are the regulators of host responses to infection and inflammation. Some cytokines such as proinflammatory cytokines, including, tumor necrosis factor-α, (TNF-α), interleukin (IL)-1, IL-6, and interferon (IFN)-γ promote inflammation and act to make disease worse whereas others such as anti-inflammatory cytokines including, IL-4, IL-6, IL-10, IL-11, and IL-13 serve to reduce inflammation and promote healing (Charles and Dinarello 2000).

In the early stage of an inflammatory response, the predominant cell type infiltrating the tissue is the neutrophil. Neutrophils release mediators such as proinflammatory cytokines, histamines and prostaglandines that attract macrophages to the site of inflammation (Goldsby et al. 2003). Activated macrophages secrete three major proinflammatory cytokines, namely IL-1, IL-6 and TNF-α, which induce many of localized and systemic changes observed in the acute inflammatory response. All three cytokines act locally, inducing coagulation and an increase in vascular permeability. IL-1 and TNF-α also act on macrophages and endothelial cells to induce production of chemokines such as IL-8 (Baumann and Gauldie 1994; Lowry 1993; Watkins et al. 1995).

Acute inflammation is generally accompanied by a systemic reaction known as the acute-phase response (Koj 1985). This response is marked by the induction of fever, increased synthesis of hormones such as adrenocorticotropic hormone (ACTH) and hydrocortisone, increased production of white blood cells and production of a large number of acute phase proteins in the liver. Many systemic acute phase effects are due to the combined action of IL-1, IL-6 and TNF-α. Each of these cytokines acts on the hypothalamus to induce a fever response. Increased levels of IL-1, IL-6 and TNF-α induce production of acute phase proteins by hepatocytes. TNF-α acts on vascular endothelial cells and macrophages to induce secretion of colony-stimulating factors (CSFs). These CSFs stimulate hematopoiesis, resulting in transient increases in the number of white blood cells needed to fight the infection (Goldsby et al. 2003).

During chronic inflammation, macrophages are accumulated (Goldsby et al. 2003). Two cytokines in particular, IFN-γ and TNF-α, play a central role in the development of chronic inflammation. Members of the interferon family (IFN-α and IFN-β) are released from virus-affected cells and confer antiviral protection on neighbouring cells. IFN-α is produced by leukocytes and IFN-β is made largely by fibroblasts. However, IFN-γ, which is released by T helper type 1 (T_h1) cells, natural killer cells and cytotoxic T (T_C) cells, is considered as proinflammatory cytokine and has a number of pleiotropic activities that distinguish it from IFN-α and IFN-β (Mannering and Deloria 1986). In addition to the release of cytokines in chronic inflammatory response and infection, the large numbers of activated macrophages and neutrophils release various hydrolytic enzymes and reactive oxygen species and nitrogen intermediates, which have potent antimicrobial activity and also are responsible for much of the damage to surrounding tissue (Goldsby et al. 2003).

The duration and intensity of the local acute inflammation response must be carefully regulated to control tissue damage and facilitate the tissue–repair mechanisms that are necessary for healing. A balance between the effects of proinflammatory and anti-inflammatory cytokines is essential in controlling the inflammatory response. Anti-inflammatory cytokines determine the outcome of inflammation by suppressing the production and the activity of TNF-α, IL-1, IL-6 and IL-8. A variety of anti-inflammatory cytokines, including IL-4, IL-5, IL-10, and IL-13, are produced by T helper type 2 (T_h2) cells (Opal and DePalo 2000).

3.2. Regulation of cytochrome P450 by inflammation

The regulation of CYP enzymes in models of infectious and inflammatory disease has been studied for 30 years, and has been described in several recent reviews (Aitken et al. 2006; Morgan 1997; Morgan 2001; Renton 2000; Renton 2001). In humans and animals, CYP and their activities are usually suppressed by infections and other inflammatory stimuli, though some are unaffected or may be induced. It is apparent that infection or inflammation can decrease metabolic clearance of CYP substrates by 20%–70% (Aitken et al. 2006). Therefore, CYP suppression can result in increased clinical toxicity of drugs with low therapeutic indices. Conversely, suppression of metabolism during an inflammatory response may lead to reduced therapeutic or toxic effects of drugs that are metabolized to pharmacologically or toxicologically active compounds (Bleau et al. 2001; El-Kadi and du Souich 1998; El-Kadi et al. 1997). These changes in CYPs have been linked to increased serum concentrations of proinflammatory cytokines such as IL-1, IL-6, TNF-α, and IFN-γ.

3.2.1. Effect of inflammation and infection on individual CYP gene expression

3.2.1.1. Human studies

A recent study in children with bacterial sepsis showed the effects of a severe bacterial infection on human drug metabolism in which the clearance of antipyrine was reduced by up to threefold, and its half-life prolonged up to five-fold. Clearnce of antipyrine by verious CYPs suggests several CYPs may be down-regulated in patients with sepsis (Carcillo et al. 2003). CYP3A4, the predominant CYP in human liver, is involved in the metabolism of approximately 40% of therapeutic agents (Guengerich

2003). Earlier study in patients with septic shock showed the clearance of midazolam as well as the formation of its metabolite, 1-hydroxymizadolam, which is catalyzed by CYP3A4, was reduced (Shelly et al. 1987). On the other hand, in children with Crohn's disease, a chronic inflammatory bowel disease, CYP3A4 and 3A5 mRNA levels increased in duodenal tissue as compared with control groups (Fakhoury et al. 2006). Haack et al. reported that infection or an inflammatory reaction cause the increase in plasma concentration of clozapine, which is metabolized by CYP1A2 and 3A4. They have shown that elevations of plasma concentration of clozapine sometimes occurred in the absence of a detectable infection. Therefore, they suggested that an inflammatory or hypersensitivity reaction to the drug itself may affect the concentration of clozapine (Haack et al. 2003).

Earlier studies showed that in asthmatic children during acute influenza and adenoviral infections, decrease in the metabolic clearance of the CYP1A2 substrate theophylline results in increase the plasma half-life of theophylline (Chang et al. 1978). Moreover, in humans CYP3A4 expression and activity also decreased following influenza immunization (Hayney and Muller 2003). In patients with hepatitis C-positive liver cirrhosis, infection with *Helicobacter pylori* causes a 60%–70% decrease in the metabolism of lidocaine to monoethylglycylxylidide, which is catalyzed by CYP1A2 (Giannini et al. 2003). However, Bleau et al. found that serum from humans with an acute upper respiratory viral infection decreased CYP activity by around 40% without any change in the amount of CYP1A1 and CYP1A2 apoproteins (Bleau et al. 2000).

3.2.1.2. Animal studies

3.2.1.2.1. Inflammation

Earlier studies reported that compounds that stimulate the release of cytokine from reticuloendothelial system can impair drug metabolism (Wooles and Munson 1971). *In vivo* stimulation of the reticuloendothelial system with dextran sulfate or latex beads decreased hepatic CYPs content, aminopyrine metabolism and AHH activities. In addition, *in vitro* stimulation of isolated Kupffer cells using the same compounds caused a decrease in CYPs content and AHH activity of hepatocytes within 30 min (Peterson and Renton 1984; Peterson and Renton 1986). Sewer et al. have reported that intraperitoneal (i.p.) administration of celite, barium sulfate ($BaSO_4$), or kaolin suppressed hepatic CYP2C11 mRNA and protein and induced CYP4A1, 4A2, and 4A3 mRNA expression while having no significant effect on CYP2E1 or 3A2. CYP4A2, 4A3, and 2E1 mRNAs were all induced in the kidney cortices of irritant-treated rats (Sewer and Morgan 1997). In rabbits, local inflammation caused by turpentine administration decrease the clearance of tolbutamide associated with a decrease in the formation of its metabolite, tolbutamide hydroxylase as well as the amount of CYP3A6 (Parent et al. 1992). It was reported that CYP3A6 is more vulnerable than CYP1A1 and CYP1A2 to the down-regulation provoked by turpentine-induced acute inflammatory reaction in rabbits (Bleau et al. 2001). In rats, inflammation induced by turpentine suppressed hepatic CYP2C11 and 2C12 mRNA levels. Transcription of CYP2C11 in male rats was reduced to 23% of control levels by turpentine (Wright and Morgan 1990).

A Study on the effect of an established model of inflammatory bowel disease (3% dextran sulfate sodium) on hepatic activities of CYP3A2, 2C11, 1A2, 2E1, and 2D2 in rats has shown that all activities except CYP2D2 activity were down-regulated by sulfate sodium treatment, and this down-regulation was selectively prevented by treatment with antibacterial drugs, polymyxin B or metronidazole, indicating the involvement of bacterial endotoxins in some of the effects (Masubuchi and Horie 2004).

Interestingly, it has been found that the CYPs were affected differently in the chronic phase of inflammation. In rats with carrageenan-induced granuloma, CYP2D6, 2E1, and 3A1 expression showed partial recovery by 6 days, while CYP2B expression and CYP2A1 activity were still strongly suppressed (Muntane-Relat et al. 1995). Decreases in CYP have been also documented in animals with chronic or end-stage renal disease (Nolin et al. 2003). Specific rat liver CYP mRNAs and proteins (CYP2C11, 3A, but not 1A2, 2C6, 2D1, or 2E1) were down-regulated in a rat model of chronic renal disease (Leblond et al. 2001).

3.2.1.2.2. Bacterial infections

In the best-studied model of infection, that of lipopolysccharide (LPS) exposure, most heptic CYPs are down-regulated, but a few are unaffected or even induced. It has been reported that Cyp1a2, 2a5, 2c29, 2e1, 3a11, 4a10, and 4a14 mRNAs in livers of LPS-treated mice were down-regulated, whereas Cyp3a13 mRNA was unaffected (Richardson and Morgan 2005). Prevoius studies had shown that an injection of LPS caused down-regulation of CYP2C11, 2C12, 2C7, and 3A2 in rats (Morgan 1989;

Morgan 1993; Sewer et al. 1996). However, the expression of CYP4A1, 4A2, and 4A3 mRNAs as well as the level of CYP4A3 protein and activity were increased in the livers of LPS-treated F344 rats (Sewer et al. 1996), but they were down-regulated in mice (Barclay et al. 1999). In rat, localized inflammatory responses induced by injection of LPS into the lateral ventricle of brain reduced CYP1A1 activity. This loss was accompanied by a concomitant loss of CYP1A1 activity in liver (Renton and Nicholson 2000). Recently, it has been reported that in contrast to the increase in hepatic CYP2E1 mRNA, a significant reduction in the catalytic activity of this enzyme was observed 24 h following the administration of LPS to the rats (Abdulla et al. 2006). In rat liver, inflammation induced by other model of infection, Freund's complete adjuvant (composed of inactivated and dried mycobacteria), also decreased total CYP contents as well as RNA levels of CYP2B, CYP2CI1, CYP3A1, and CYP2E1, protein contents of CYP2B, CYP2C11, and CYP2E1 or catalytic activities of CYP2C6, CYP2C11, and CYP2E1 (Projean et al. 2005).

Early studies on the effect of infection with the gram-positive bacterium *Listeria monocytogenes* in mice resulted in the reduction in CYP content and AHH activity of hepatic microsomes, as well as a decrease in aminopyrine demethylation and an increase the half-life of theophylline (Azri and Renton 1987). Armstrong and Renton have also reported that infection of mice with *Listeria. monocytogenes* suppressed the constitutive expression of Cyp2d6 mRNA (Armstrong and Renton 1993). Infection of guinea pigs with live *Mycobacterium tuberculosis* was shown to cause decreases in hepatic CYP content and hepatic and pulmonary drug-metabolizing activities (Batra et al. 1987).

Infection with the respiratory pathogen *Actinobacillus pleuropneumoniae* decreased clearances of antipyrine, caffeine and acetaminophen in pigs 24 h after inoculation and this was associated with decreased microsomal metabolism of aniline, ethoxyresorufin, pentoxyresorufin, and testosterone at the 2β, 6β, 11α, 15α, and 15β positions and decreased CYP3A mRNA (Monshouwer et al. 1995a; Monshouwer et al. 1995b).

The induced expression of most CYPs can also be down-regulated during inflammation. However, the observed effect depends on the time after inflammatory stimulus, the nature of the stimulus, the animal species and the magnitude of the inflammatory response (Morgan 1997). Co-treatment of isolated rat hepatocytes with LPS and phenobarbital or 3-MC resulted in a strong down-regulation (85%) of phenobarbital-induced CYP2B1 whereas 3-MC-induced CYP1A1 was only weakly affected (15%) (Milosevic et al. 1999). Li-Masters and Morgan found that LPS suppressed the expression of phenobarbital-induced Cyp2b expression in mice with similar effects in rats (Li-Masters and Morgan 2001). LPS inhibited the mRNA expression of Cyp2b10 and Cyp2b9 at 6 and 12 h after treatment, with the maximum effect at 12 h (Li-Masters and Morgan 2001). In addition, βNF-treated mice infected with *L. monocytogenes* showed a transient induction of Cyp1a1 mRNA, protein and activity at 12 to 24 h after infection, followed by a profound decrease in these parameters (Armstrong and Renton 1993).

3.2.1.2.3. Viral infection

As early as 1963, Kato et al. reported that murine hepatitis virus impaired hepatic metabolism of hexobarbital and strychnine (Kato et al. 1963). It was found that Newcastle disease virus, encephalomyocarditis virus and influenza virus decreased hepatic CYP contents and activities (Corbett and Nettesheim 1973; Renton 1981; Singh and Renton 1981). A recent study has shown that infection of male rats with a model recombinant adenoviral vector caused a dose-dependent down-regulation of CYP3A mRNA, protein, and activity, whereas CYP2C11 protein and activity, but not mRNA, were increased at low viral doses (Callahan et al. 2005). Previously, Leeson and Renton have shown that the antiviral drug tilorone, which induces IFN, caused a decrease in the content of CYPs (Leeson et al. 1976; Renton and Mannering 1976). IFN inducers or IFNs suppressed the expression of several CYPs, including CYP1A1, 1A2, 2C11, 2C12, 2E1, and 3A2, in rat (Cribb et al. 1994; Cribb and Renton 1993; Morgan 1991; Sakai et al. 1992). Some other studies have reported that the expression of Cyp1a1, 1a2, 2b10, 2e1, and Cyp2c6 in the mouse was down-regulated by IFNs and IFN inducers (Anari et al. 1995; Stanley et al. 1991). In addition, in rats, the induced expression of CYP3A1 (by troleandomycin or pregnenolone 16α-carbonitrile), CYP1A (by pyridine or βNF) and CYP4A (by clofibrate) were also suppressed by IFN inducers (Cribb et al. 1994; Delaporte et al. 1993; Knickle et al. 1992). IFNs did not affect the expression of induced CYP3A1 in the female rat, or Cyp1a1, Cyp1a2, Cyp2c6 or Cyp2b1 in the mouse (Abdel-Razzak et al. 1994; Clark et al. 1996).

3.2.1.2.4. Parasitic infections

Infection of rats with the malaria parasite *Plasmodium berghei* decreased the clearance of metronidazole and caffeine but not antipyrine (Kokwaro et al. 1993a; Kokwaro et al. 1993b). The hookworm parasite, *Ancylostoma ceylanicum*, also caused a decrease in hepatic CYP content and decreases in aminopyrine demethylase and AHH activity (Tekwani et al. 1990). In addition, infection of rats and sheep with the liver fluke parasite *Fasciola hepatica* suppressed the expression of CYP2C11, 2A, 2B, 2C7 and 3A protein while CYP2E1 expression was unaffected (Biro-Sauveur et al. 1994; Galtier et al. 1985).

3.2.2. Role of cytokines

3.2.2.1. Cytokines and cytochrome P450

The effects of cytokines on expression of CYPs have been studied extensively in cultured hepatocytes during inflammation and infection. In LPS-mediated infection, LPS is recognized by toll-like receptor (TLR)-2 and 4, which express by hepatocytes. Activated TLRs induce production of several proinflammatory cyokines including TNF-α, IL-1 and IL-6 which are important in the modulation of expression of various CYPs during infection (Schwabe et al. 2006). It has been reported that IL-4 increased CYP2E1 mRNA expression, without affecting CYP1A1 activity (Abdel-Razzak et al. 1993). Other studies have shown that cytokines strongly repress the inducibility of CYP1A1, 1A2 and 3A4 genes at a transcriptional or a post-transcriptional level. IL-6 had the most potent effect on CYP3A4, whereas TNF-α was the most potent with CYP1A genes (Muntane-Relat et al. 1995). Both the basal and 3-MC- or benzo[a]pyrine-induced expression of

CYP1A in human hepatocytes were attenuated by TGF-β (Abdel-Razzak et al. 1994). Moreover, in human hepatocytes, IFN-γ suppressed CYP1A2 and CYP2E1 mRNA levels and CYP1A1 activity (marker for CYP1A1 activity) but had no effect on CYP3A and epoxide hydrolase mRNAs. Table 3.1 shows the effects of cytokines on basal and inducible expression of different CYPs in human hepatocytes.

Table 3.1. Effect of cytokines on the expression of cytochrome P450 enzymes in human hepatocytes

CYP	Inducer	Cytokine	Effect	References
1A1	3-MC	TGFβ, IL-6	↓	(Abdel-Razzak et al. 1994; Fukuda and Sassa 1994)
	βNF	IL-6, TNF-α	↓	(Muntane-Relat et al. 1995)
	TCDD	IL-1β	↓	(Barker et al. 1992)
1A2	none	Oncostatine M, IL-6, IFN-γ, TNF-α, TGFβ	↓	(Abdel-Razzak et al. 1994; Abdel-Razzak et al. 1993; Guillen et al. 1998)
	3-MC	Oncostatine M, TGFβ, IFN-γ	↓	(Abdel-Razzak et al. 1993; Donato et al. 1997; Guillen et al. 1998)
	βNF	IL-6, TNF-α	↓	(Muntane-Relat et al. 1995)
	TCDD	IL-1β	↓	(Barker et al. 1992)
2A6	none	Oncostatine M, IL-6, IFN-γ	↓	(Guillen et al. 1998)
	3-MC	IFN-γ	↓	(Donato et al. 1997)
2B6	none	Oncostatine M, IL-6, IFN-γ	↓	(Guillen et al. 1998)
	Phenobarbital/ Bilirubin	IL-1β	↓	(Assenat et al. 2004)
	3-MC	IFN-γ	↓	(Donato et al. 1997)
2C	none	TNF-α	↓	(Abdel-Razzak et al. 1993)
	Phenobarbital/ Bilirubin	IL-1β	↓	(Assenat et al. 2004)

CYP	Inducer	Cytokine	Effect	References
2E1	none	IL-2, IL-6, TNF-α, IFN-γ	↓	(Abdel-Razzak et al. 1993)
	none	IL-4	↑	(Abdel-Razzak et al. 1993)
3A4	none	Oncostatine M, IL-1, IL-6, IFN-γ, TNF-α, TGFβ	↓	(Abdel-Razzak et al. 1993; Donato et al. 1997; Guillen et al. 1998)
	Phenobarbital/ Bilirubin	IL-1β	↓	(Assenat et al. 2004)
	none	IFN-γ	→	(Abdel-Razzak et al. 1993)
	3-MC	IFN-γ	↓	(Donato et al. 1997)
	dexamethazone	Oncostatine M	↓	(Guillen et al. 1998)
	rifampicin	IL-6	↓	(Muntane-Relat et al. 1995)
	rifampicin	IL-1, TNF-α	→	(Muntane-Relat et al. 1995)

Earlier studies have reported that injection of IL-1 and TNF-α to mice caused a significant decrease in CYP1A1 activity (Ghezzi et al. 1986a; Ghezzi et al. 1986b). Study on rat hepatocytes has shown that low concentrations of recombinant IL-1 suppressed the TCDD-dependent accumulation of both CYP1A1 and 1A2 mRNAs in a concentration-dependent manner while IL-6 had no effect. IL-1 also rapidly suppressed the transcription rate of CYP1A1 and 1A2 in TCDD-treated hepatocytes. The close correspondence between the reductions in CYP1A1 and 1A2 transcription rates and mRNA levels suggest that IL-1 suppresses the induction of these mRNAs principally through a transcriptional mechanism (Barker et al. 1992). It has also been reported that IL-1β and TNF-α, but not IL-6, inhibited the dibenzo[a,h]anthrancene-mediated induction of CYP1A1 activity in isolated rat astrocytes (Nicholson and Renton 2002). The administration of a single dose of IL-1α to rats suppressed the total hepatic CYP, as well as metabolism of benzphetamine, ethoxycoumarin, aminopyrine, debrisoquine and bufuralol (Kurokohchi et al. 1992). The decrease in debrisoquine and bufuralol hydroxylase activities, characteristics of the CYP2D enzyme, is accompanied by a proportional reduction in CYP2D mRNA and protein levels (Kurokohchi et al. 1992). It has been reported that in rats, three i.p. injections of IL-1β suppressed the expression of mRNA and protein of female-specific CYP2C12, while a single injection, presumably due to the short biological half-life of IL-1β, had no effect (Wright and Morgan 1991). Three daily doses of IL-1β reduced CYP3A2, 2C11, and 1A1 activities, in male rats, but not CYP1A1 activity in female rats (Ferrari et al. 1993a). The administration of TNF-α to the rats decreased CYP2C11 and CYP3A2 mRNA and protein whereas CYP2A1 protein levels were not affected (Nadin et al. 1995). IL-6 injection to rats reduced CYP2C11 mRNA

and protein, as well as CYP2E1 mRNA, but did not affect CYP2E1 protein levels or CYP3A2 expression (Morgan et al. 1994).

Interestingly, studies on mice with targeted deletions of the genes for cytokines or cytokine receptors demonstrated that absence of the IL-6, IL-1β genes or the p55 and p75 receptors for TNF-α had no effects on the down-regulation of several CYP genes in the LPS model (Ashino et al. 2004; Siewert et al. 2000; Warren et al. 1999). This may be explained by redundant function of the various cytokines released during LPS-induced inflammation (Siewert et al. 2000). In contrast to the LPS model, deletion of IL-6 gene blocked the suppression of Cyp3a11 and 2c29 in mice treated with tuberculosis vaccine, and of Cyp1a2, 2a5, and 3a11 in mice treated with turpentine (Ashino et al. 2004; Siewert et al. 2000). In mice infected with *Bacillus Calmette-Guérin*, IL-1α/β gene deficiency had no effect on Cyp3a11 or 2c29 down-regulation, whereas TNF-α deficiency had partial effects (Ashino et al. 2004). These observations revealed an *in vivo* role for IL-6 and perhaps TNF-α in suppression of specific CYP genes in non-LPS model of inflammation. The role of IL-6 was also supported by the studies on rabbit hepatocytes cultured with serum from turpentine-treated rabbits or humans with an upper respiratory viral infection. Immuno-neutralization of these sera with cytokine antibodies showed that IL-6 is involved in down-regulation of CYP1A1, 1A2, and 3A6 mRNAs and proteins as well as CYP1A1 activity (Bleau et al. 2000; Bleau et al. 2003).

3.3. Regulation of phase II xenobiotic metabolizing enzymes by inflammation

The information concerning regulation of phase II xenobiotic metabolizing enzymes during inflammation is limited and requires much more study to determine which isoforms are modulated by inflammation and the mechanisms involved.

After administration of LPS in rats and isolated perfused rat liver, minor decreases in hepatic UGT activities were reported, however after injection of individual cytokines such as recombinant human IL-6 in rats no effect on UGTs was observed (Banhegyi et al. 1995; Chen et al. 1992). In addition, administration of endotoxin in rats significantly decreased UGTs activity after 24 h, and this activity remained lower than control for 72 h (Watson et al. 1999). In rats treated with turpentine, hepatic UGT1A6 was unchanged, whereas hepatic UGT2B3 mRNA and activity were decreased at 24 h (Strasser et al. 1998). In isolated rat hepatocytes, IL-6, but not IL-1α, down-regulated UGT1A1 and 2B3 mRNA expression (Strasser et al. 1998). However, in rat astrocytes, LPS up-regulated UGT1A6 mRNA and activity (Heurtaux et al. 2006). In mice, after LPS treatment and *Citrobacter rodentium* infection, hepatic expression of Ugt1a1, 1a9, and 2b5 mRNA was down-regulated, whereas Ugt1a2 and 1a6 mRNAs were unchanged (Richardson et al. 2006). In isolated pig hepatocytes, IL-1α, IL-6 and TNF-α inhibited glucuronidation (Monshouwer et al. 1996). In human prostate cancer cell lines, LNCaP, treatment with IL-1α for 6 days suppressed testosterone glucuronidation by 70%, and specifically suppressed UGT2B17, but not UGT2B15, mRNA and protein, (Levesque et al. 1998). In a single study with human liver biopsies, it has been shown that decreased

UGT1A4, 2B4, and 2B7 mRNA levels correlated with increased inflammation (Congiu et al. 2002).

With respect to GST, after *in vivo* LPS administration, differential effects on basal GST isozyme expression have been reported. For example, rGSTA2 and A3 were significantly down-regulated, whereas rGSTM1 and M2 were less affected (Buetler 1998; Choi and Kim 1998). In rats, after administration of LPS (1 mg/kg), constitutive hepatic GST mRNA and protein was unaffected at either 6 or 24 h. However, LPS inhibited the oltipraz-inducible expression of of GSTA1/2, M1, and P1 mRNAs at 24 h (Maheo et al. 1998). In rat liver, LPS down-regulated allyl disulfide- and oltipraz-mediated induction of rGSTA2, A3, M1, and M2 mRNAs by 50 – 90% (Choi and Kim 1998). Studies of the exposure of rats to endotoxin showed conflicting results with GST activity. It has been reported that in rat hepatocytes, IL-1β down-regulated rGSTA2 and M1 mRNAs, however induced rGSTP1. The decline in rGSTM1 mRNA was due to accelerated degradation of the mRNA (Maheo et al. 1997). In human study, after a three-day treatment of human hepatocytes, no reproducible effects of IL-6 demonstrated on GSTA1 and/or A2 or M1 mRNA levels. In contrast, after IL-4 treatment, GSTA1 and/or A2 mRNAs and GSTA1 and A2 proteins were reproducibly increased (Langouet et al. 1995). In human prostate, simple atrophy and post-atrophic hyperplasia-proliferative lesions associated with chronic inflammation, which have been termed "proliferative inflammatory atrophy", produced elevated levels of GSTP1 and GSTA1 (Parsons et al. 2001)

4. MECHANISMS OF REGULATION OF XENOBIOTIC METABOLIZING ENZYMES DURING INFLAMMATION

4.1. Mechanisms of regulation of cytochrome P450 during inflammation

4.1.1. Cellular signalling pathways

4.1.1.1. Reactive oxygen species (ROS)

Oxygen, due to its electronic configuration, is prone to gain electrons and becomes a potent oxidant. During the respiration process, O_2 is constantly reduced by four electrons to yield water. However, the incomplete reduction leads to the formations of free radicals, such as superoxide, hydrogen peroxide, and hydroxyl radical which are known as ROS (Morel and Barouki 1999). ROS production stimulated by infection and inflammation was first described in phagocytic cells including, neutrophils and macrophages (Forman and Torres 2001). There are many other different sources by which the ROS are generated including, oxidative phosphorylation in mitochondria, biotransformation of exogenous and endogenous compounds involving the hepatic CYP enzyme, enzymatic reactions catalyzed by NADPH oxidase and xanthine oxidase, oxidoreduction reactions involving eicosanoids production, ethanol metabolism in hepatocytes, and lipid peroxidation of unsaturated fatty acids (Djordjevic 2004). Once ROS were produced, they are removed by antioxidant defences, including catalse, glutathione peroxidase, and superoxide dismutase. During inflammatory responses or infections, the production of a number of ROS is part of the response. In this regard, proinflammatory cytokines such as IFN-γ, IL-1β, and IL-6 can activate xanthine oxidase to generate superoxide (Ghezzi et al. 1985). TNF-α also causes a disruption of the

mitochondrial electron transport chain, resulting in intracellular ROS release (Schulze-Osthoff et al. 1993).

There is some evidence that production of ROS may contribute to the inhibition of CYP activity. It has been reported that serum from rabbits with a turpentine-induced inflammatory response or from humans with a viral infection caused a marked loss in total CYP content and CYP-dependent activities in isolated rabbit hepatocytes, and this was accompanied by a significant increase in lipid peroxidation (El-Kadi et al. 1997). This loss in CYP and in theophylline metabolism can be prevented by antioxidants, and is enhanced when antioxidant-producing enzymes are inhibited (El-Kadi et al. 2000). Furthermore, the vitamin E analog Trolox inhibited the decrease in total hepatic CYP caused by cecal ligation and puncture in rats, and differentially inhibited the decreases in hepatic CYP2B1, 1A2, and 2E1 activities (Park and Lee 2004). Several studies also support a role of ROS in CYP down-regulation by LPS treatment. Cyp3a11 down-regulation by LPS in mouse liver and placenta was inhibited by treatment with inhibitors of xanthine oxidase and NADPH oxidase, antioxidants, and free radical spin traping agent (Chen et al. 2005; Xu et al. 2004). Additional experiments revealed that LPS-initiated lipid peroxidation in mouse placenta were also attenuated by antioxidant and free radical spin traping agent (Chen et al. 2005; Xu et al. 2004).

H_2O_2 has been shown to mediate the loss of hepatic CYPs such as CYP1A1 and CYP1A2 mRNA in isolated rat hepatocytes (Barker et al. 1994). The possibility that ROS like H_2O_2 might directly decrease the CYP1A1 activity was proposed yeas ago. It has

been reported that H_2O_2 can directly interact with the enzyme-associated Fe^{2+} leading to heme destruction and enzyme inactivation (Karuzina and Archakov 1994). On the other hand, H_2O_2 can act as a second messenger leads to the inactivation and/or suppression of selected CYP isoforms. ROS, specifically, O_2^- and H_2O_2 play an important role as stimulators of protein tyrosin kinase C, protein kinase A, and mitogen-activated protein kinases (MAPK). Activation of kinases, which can phosphorylate CYP, results in inactivation of CYP (Bae et al. 1997; Boyer et al. 1995; Goldstone and Hunt 1997; Lowe et al. 1998; Suzuki et al. 1997).

A well-documented transcription factor, which is activated by ROS, is NF-κB (Legrand-Poels et al. 1997). LPS and proinflammatory cytokines such as TNF-α and IL-1β are known to induce NF-κB, which plays a direct role in TNF-α - and LPS-induced down-regulation of *Cyp1a1* expression (Ke et al. 2001). It has been reported that either ROS-mediated activation of NF-κB or TNF-α- and LPS-mediated suppression of CYP1A1 expression can be prevented by antioxidants. Thus, the increase in ROS production by TNF-α and LPS suppresses the CYP1A1 expression, possibly through the activation of NF-κB activity (Janssen-Heininger et al. 2000). Activator protein-1 is another transcription factor, which is also activated by ROS (Sen and Packer 1996). The role of activator protein-1 to modulate CYP1A1 and 1A2 expression is controversial. It has been reported that TGF-β-mediated down-regulation of CYP1A1 was dependent on the activation of activator protein-1 (Abdel-Razzak et al. 1994) whereas, other evidence suggests that activator protein-1 is involved positively in the up-regulation of CYP1A1 and 1A2. For example, TCDD induces a significant increase in activator protein-1

activity as well as enhances the expression of CYP1A1 and 1A2 (Nebert et al. 1993). Also, CYP1A2 induction by 3-MC involves activator protein-1 binding to its promoter (Quattrochi et al. 1998). In contrast to NF-κB and activator protein-1 which are activated by ROS, nuclear factor I/CCAAT box transcription factor (NFI/CTF-1) are repressed by oxidative stress. In the response of proinflammatory cytokines such as TNF-α that induce ROS production, the repression of CYP1A1 mRNA and the *CYP1A1* gene promoter, which is a target of NFI/CTF-1 are also mediated by NFI/CTF-1 (Morel et al. 2000).

4.1.1.2. Nitric oxide (NO)

Nitric oxide is biosynthesized from the amino acid L-arginine by at least three different isoforms of NO synthase including neuronal NO synthase (NOS1), inducible NO synthase (NOS2), and endothelial NO synthase (NOS3) (Southan and Szabo 1996). The resulting NO may quickly react with molecular oxygen and water to form a variety of end products, including nitrite (NO_2^-), nitrate (NO_3^-), and S-nitrosothiols (RSNO) (Moncada et al. 1991; Nathan 1992). NO can also react with superoxide to yield a strong biological oxidant, peroxynitrite (Murphy et al. 1998). Activation of different cell types such as macrophages, endothelial cells, fibroblasts and hepatocytes with proinflammatory cytokines results in the induction of a distinct isoform of NO synthase expression, NOS2, followed by the production of NO (Xie et al. 1992). In hepatocytes, NOS2 is induced during endotoxemia or in the presence of specific combinations of cytokines and LPS or supernatant from LPS-activated Kupffer cells but not turpentine-evoked inflammation. However, hind limb injection of turpentine primes the hepatocytes to express NOS2

subsequently in response to cytokines or endotoxins (Curran et al. 1990; Geller et al. 1994).

It has been reported that NO directly binds to hemeproteins and causes a partially reversible inhibition of the CYP while the irreversible inhibition may be due to the reaction of other NO species such as peroxynitrite, with critical amino acid residues like tyrosine (Minamiyama et al. 1997; Wink et al. 1993). It is worth noting that effects of NO on CYP may vary depending on the isoform involved. For example, Vuppugalla and Mehvar have reported that in contrast to significant suppression of CYP2B1, 2C11, 3A2 activity by NO, the effect of NO on CYP2D1 activity was minimal. They have suggested that the lack of the effect of NO on CYP2D1 may be due to the lack of tyrosine residues at critical sites (Minamiyama et al. 1997; Vuppugalla and Mehvar 2004). However, it has been shown that NO is capable of inhibiting *CYP2D6* reporter gene transcription by regulating the activity of transcription factors, including hepatocyte nuclear factor 4 and NF-κB, due to cysteine-nitrosylation of the DNA binding domains of hepatocyte nuclear factor 4 and NF-κB p50 (Hara and Adachi 2002; Marshall and Stamler 2001; Matthews et al. 1996; Vossen and Erard 2002).

The role of NO in the regulation of hepatic CYP expression in inflammation has been the subject of debate. Some of the evidence for the participation of NO in down-regulation of CYP mRNAs and proteins has been obtained with CYP2B enzymes. Administration of N-nitro-L-arginine methyl ester, a NOS2 inhibitor, to rats treated with phenobarbital and LPS blocked the LPS-mediated down-regulation of CYP2B1/2

activity, mRNA and protein (Khatsenko et al. 1997). Inhibitors of NOS2 also blocked the suppression of CYP2B1/2 proteins mediated by a cytokine combination consisting of TNF-α and IL-1β, in primary cultures of rat hepatocytes (Carlson and Billings 1996), although the modulation of CYP2B1/2 mRNAs in this study was not reported. In addition, in rat hepatocytes, inhibition of NOS2 attenuated the decreases in the levels of CYP2B, 2C11 and 3A2 protein evoked by IL-1β and TNF-α (Carlson and Billings 1996). It has also been shown that LPS-mediated decrease of hepatic Cyp1a and Cyp2b in mice were associated with NOS2 induction, followed by NO overproduction and that the response was prevented by NOS inhibitors (Khatsenko et al. 1998; Khatsenko et al. 1993). However, Ferrari et al., have shown that a rapid NO-dependent loss of CYP2B1/2 protein in primary cultures of rat hepatocytes occurred at high LPS concentrations while a slower NO-independent pathway suppressed CYP2B1 mRNA at lower LPS concentrations (Ferrari et al. 2001). On the other hand, a large number of studies have ruled out the possible role of NO in the down-regulation of CYP expression by finding that the down-regulation of the majority of hepatic CYP proteins and mRNAs by LPS or cytokines is not affected by deletion of the NOS2 gene or by inhibitors of NOS2 enzyme. In pig hepatocytes, Monshouwer et al. demonstrated that inhibition of NOS2 by N-G-nitro-L-arginine had no effect on the inhibition of CYP in response to IL-1, IL-6, or TNF-α (Monshouwer et al. 1996). In rat hepatocytes cultured on Matrigel, down-regulation of CYP2C11 mRNA or protein expression in response to IL-1β and LPS was not affected by inhibition of NO production (Sewer and Morgan 1997). In mice lacking NOS2 gene and incapable of producing NO, the administration of LPS caused a down-regulation in Cyp2c, Cyp3a, and Cyp2e1 (Sewer et al. 1998). Hodgson and Renton have also reported

that treatment of mice with N^{ω}-nitro-L-arginine did not attenuate the decrease in total Cyp or Cyp1a or Cyp2e1 activity produced by interferons (Hodgson and Renton 1995).

4.1.1.3. Phosphorylation

Some *in vitro* and *in vivo* studies have shown that CYP isoforms are selectively phosphorylated. The protein kinase A recognition motif Arg–Arg-X–Ser is only active in some of CYP isoforms such as CYP2B1/2B2 and CYP2E1 (Oesch-Bartlomowicz and Oesch 2003). Although the phosphorylation of CYP2B1 in rat hepatocytes results in decrease CYP2B1 activity in 4 min the amount of CYP2B1 protein remained unchanged for up to 3 h. These results showed that phosphorylation leads to decrease CYP2B1 activity without increasing the rate of its degradation (Oesch-Bartlomowicz et al. 2001). With respect to CYP2E1, one study reported that in rat hepatocytes phosphorylayion of CYP2E1 increases protein degradation (Eliasson et al. 1992). However, the other study showed that treatement of Chinise hamster lung fibroblast cells with protein kinase A-stimulating agents reduced CYP2E1 activity without increasing the rate of protein degradation (Oesch-Bartlomowicz et al. 1998).

In addition to CYP2Bs and CYP2E1, other studies have shown that activation of different kinases including, Janus-associated kinases (JAK), extracellular signal-related kinase (ERK1/2), double stranded RNA-dependent kinases, protein kinase C, and mitogen-activated protein kinases (MAPK), which can phosphorylate CYP, results in inactivation of other CYP isoforms. Various protein kinase inhibitors have been shown to

attenuate the reduction of total CYP content and the inhibition of CYP1A1, 1A2 and 3A6 activities by inflammatory sera in rabbit hepatocytes (Levitchi et al. 2004).

4.1.2. Transcriptional regulation

In most cases, down-regulation of CYP activities and protein levels are accompanied or preceded by decreases in the respective CYP mRNAs (Renton and Nicholson 2000; Sewer and Morgan 1997). Recently, it has been reported that the transcription of CYP2C11, 3A2, and 2E1 is suppressed to 20%, 30%, and 10% of control, respectively, within 1–2 h of LPS treatment in rats (Cheng et al. 2003). However, other studies have shown that LPS acts at two different levels to regulate hepatic CYP2E1: a transcriptional level to increase CYP2E1 mRNA expression and a post-transcriptional level to regulate CYP2E1 protein and activity (Abdulla et al. 2006). With respect to Cyp1A1/1A2, the transcription rate of hepatic *CYP1A1/1A2* genes decreased along with increasing the degradation of hepatic CYP1A1/1A2 mRNA upon treatment with the Interferon (IFN)-α/β inducer polyinosinic acid-polycytidylic acid in rat. These results support the involvement of both transcriptional and post-transcriptional mechanisms in down-regulation of CYP1A1/1A2 mediated by IFN inducers (Delaporte and Renton 1997). On the other hand, the observations that AhR/ARNT interact with a number of diverse transcription factors implicate an additional mechanism by which the AhR signalling pathway may alter *CYP1A1* gene expression during inflammation. The AhR and ARNT have been found to interact with components of other signalling pathways such as NF-κB (Tian et al. 1999). It is well known that LPS and TNF-α activate the NF-κB (Ghosh et al. 1998). Interestingly, it has been demonstrated that there is a mutual

inhibitory interaction between the AhR and the NF-κB signalling pathways, suggesting that NF-κB may play a direct role in mediating the suppression of induced transcription of CYP1A1 and 1A2 by inflammatory stimuli (Tian et al. 1999). Furthermore, it has been noted that coactivator and corepressor proteins which are involved in histone-acetylation mediate the cross-interactions between the AhR and NF-κB signalling pathway (Ke et al. 2001). Histone-acetylation of the *Cyp1a1* promoter, an important initial step for gene activation, found to be suppressed by treatment with TNF-α or LPS through activation of NF-κB. It is conceivable that the cross-interactions between the AhR and NF-κB signalling pathways also converge upon these coactivators and corepressors. Competition between ligand-AhR/ARNT complexes and the NF-κB RelA subunit for binding of coactivators and corepressors could affect the level of transcriptional activation seen in these two pathways (Ke et al. 2001).

4.1.3. Post-transcriptional regulation

4.1.3.1. RNA turnover

Not much evidence is available for or against stimulation of RNA degradation as a mechanism for CYP down-regulation by cytokines. Recently, Hakkola et al. have suggested that the loss of CYP2E1 in response to cytokines involves changes to the stability of mRNA along with a reduced level of transcription (Hakkola et al. 2003). In rat hepatocytes treated with monocyte-conditioned medium, CYP1A1 and 1A2 mRNA levels declined more rapidly from their corresponding controls than in hepatocytes incubated with the transcription inhibitor actinomycin D (Barker et al. 1992). These differences can attribute not only to an inhibition of RNA turnover by actinomycin D but

also to IL-1-stimulated degradation (Barker et al. 1992). Also, evidence exists that the IFN inducer-mediated loss in CYP1A1/1A2 in rat involves an increase in mRNA degradation in addition to transcriptional blockade, suggesting the contribution of both transcriptional and post-transcriptional mechanisms to the loss of CYP1A1/1A2 mediated by IFN inducers (Delaporte and Renton 1997).

4.1.3.2. Enzyme inhibition

In rat hepatocytes treated with IFN-γ, down-regulation of CYP3A2 protein to about 30% of control and decrease in the corresponding testosterone 6β–hyroxylase activity to about 3% indicates the inhibition of the activtiy of the remaining enzyme (Tapner et al. 1996). However, in rats treated *in vivo* with IFN-α, the CYP1A1 and 2B activities were suppressed without any detectable change in the corresponding proteins (Stanley et al. 1991). A similar lack of correspondence between CYP1A1 and 2B activities and protein levels was seen in arthritic rats with or without IL-1 treatment (Ferrari et al. 1993b). It has also been reported that turpentine-induced acute inflammatory reaction or serum of humans with a viral infection decreased CYP content and activity without affecting the amount of CYP1A1 and 1A2 (El-Kadi et al. 2000).

4.2. Mechanisms of regulation of phase II xenobiotic metabolizing enzymes during inflammation

Mechanisms of regulation of phase II xenobiotic metabolism enzymes during inflammation are not well understood and there are few published studies. It has been suggested that down-regulation of phenobarbital- or bilirubin-induced expression of

GSTA1/A2 by IL-1β in human hepatocytes (HepG2) cells may be due to down-regulation of constitutive androstane receptor, which mediates the induction of these genes (Assenat et al. 2004). It has also been shown that recombinant human IL-1β down-regulated the expression of GSTA1/A2 at mRNA, protein, and activity levels in a dose-dependent manner in human colonic epithelial (Caco 2) cells. In IL-1β-treated cells, GSTA1/A2 mRNA half-life was similar to control, suggesting that IL-1β has no effect on mRNA stability. Moreover, deletion of a hepatocyte nuclear factor 1 site abolished the IL-1β-mediated repression of *GSTA1/A2* promoter activity. Based on these results, Romero et al., suggested that IL-1β suppresses GSTA1/A2 expression in human colonic epithelial (Caco 2) cells by a transcriptional mechanism involving an hepatocyte nuclear factor 1 site (Romero et al. 2002). Moreover, IL-6, in the presence of dexamethasone, significantly down-regulated GSTA2, GSTM1, and GSTM2 expression after 48 h in primary cultures of rat hepatocytes (Voss et al. 1996). It has been reported that *in vitro* IL-6 and dexamethasone together does not suppress GSTA2 expression by decreasing the binding of hepatocyte nuclear factor 1 to the *GSTA2* promoter in rat hepatocytes (Voss et al. 2002). However, a novel negative regulator of GSTA2 transcription (IL6DEX-NP), which appeared only in the presence of both IL-6 and dexamethasone binds to a promoter region adjacent to the hepatocyte nuclear factor 1 site of *GSTA2* and decreases its expression (Voss et al. 2002). On the other hand, *in vivo* in rats following the administration of LPS, the kinetics of *GSTA2* gene expression and of DNA binding activity of IL6DEX-NP and hepatocyte nuclear factor 1 to a *GSTA2* promoter region has shown that decrease of hepatocyte nuclear factor 1-binding activity at 3 h leads to a rapid decrease in GSTA2 mRNA levels. After 24 and 48 h, there is an increase in IL6DEX-NP-

binding activity that continues to exert a negative effect on GSTA2 expression (Whalen et al. 2004).

HYPOTHESIS AND OBJECTIVES

The AhR plays a central role in the toxicity of numerous polynuclear aromatic hydrocarbons such as TCDD, combustion products, and phytochemicals by controlling the expression of some phase I and II XMEs responsible for their metabolism (Mandlekar et al. 2006). The AhR also mediates numerous pathophysiological abnormalities, which accompany by inflammation and appear to be species- as well as organ- or cell type-specific. These abnormalities include chloracne, thymic atrophy and immune dysfunction, hepatic damage and steatosis, gastric epithelial hyperplasia, embryonic teratogenesis, and several types of cancer (Poland and Knutson 1982). However, the molecular mechanisms underlying most aspects of these toxic responses as well as biological functions of the AhR are currently unknown. A better understanding of this phenomenon at the molecular level is critical for addressing this problem.

In humans and animals, infections and other inflammatory stimuli cause changes in the activities and the expression of various forms of hepatic and extrahepatic CYP such as CYP1A1, 1A2 and 2B1, which are regulated by AhR. These changes in CYPs have been linked to increased serum concentrations of proinflammatory cytokines (Chapter 3). In animals, LPS or turpentine administration has been shown to down-regulate CYP1A1 and 1A2 (Bleau et al. 2000; Kurdi et al. 1999). *In vitro*, IL-6 repressed CYP1A1, CYP1A2, and CYP3A mRNA in human hepatoma cells (Fukuda et al. 1992). IL-1β, IL-6, and TNF-α inhibited CYP1A2, CYP2D, CYP2E1, and CYP3A mRNA and related enzyme activities in human primary hepatocytes (Abdel-Razzak et al. 1993). Furthermore, inhibition of PAH-mediated induction of CYP1 expression by cytokines and

growth factors has also been demonstrated in different cell systems *in vitro*. For example, PAH-induction of CYP1A mRNA expression is inhibited by IL-1β, IL-6, and TNF-α (Barker et al. 1992; Fukuda and Sassa 1994; Muntane-Relat et al. 1995). Although TNF-α and LPS are known to suppress the expression and activites of CYP there is limited knowledge about their effect on the other AhR-regulated genes such as GSTA1 and NQO1 and the underlying mechanisms remnain unknown. Therefore, it is important to investigate the cellular and molecular processes that contribute to the modulation of AhR regulated genes during inflammation.

Inflammation induces NOS2 that results in the production of NO in hepatocytes, macrophages, and endothelial cells in picomolar to nanomolar ranges (Moncada et al. 1991; Nathan 1992). The role of NO on the down-regulation of CYP during inflammation is controversial. Although some studies using whole animals (Khatsenko and Kikkawa 1997; Khatsenko et al. 1998), microsomes (Minamiyama et al. 1997; Wink et al. 1993) or hepatocytes (Carlson and Billings 1996; Stadler et al. 1994) reported the close relationship between the inflammation-mediated suppression of CYPs and the production of NO, several studies using hepatocytes and whole animals have reported that NO is not required for the down-regulation of CYP by LPS or cytokines (Sewer et al. 1998; Sewer and Morgan 1997; Sewer and Morgan 1998). Support for NO-independent suppression of CYP was also provided by both *in vitro* and *in vivo* studies which reported that NOS inhibition had no effect on cytokine-mediated decreases in CYP-catalyzed activities (Hodgson and Renton 1995; Monshouwer et al. 1996). With respect to phase II enzymes, it has been reported that NO elicits a signal that presumably induces NQO1

(Dhakshinamoorthy and Porter 2004). However, little is known about the role of NO on the down-regulation of NQO1 mediated by inflammation. Therefore, it is important to examine the role of NO in the modulation of AhR-regulated genes during inflammation. For this purpose, in the experiments reported in this thesis, we tested the hypotheses that inflammation modulates AhR-regulated genes such as CYP1A1, GSTA1 and NQO1 in an AhR-dependent manner and NO is involved in the modulation of AhR-regulated genes during inflammation. To examine these hypotheses, the specific objectives of this study were 1) to assess the effects of inflammation and the interaction between inflammation and the AhR in the regulation of AhR-regulated genes; 2) to develop a sensitive and accurate HPLC method to measure picomolar levels of NO in murine hepatoma Hepa 1c1c7; and 3) to investigate the role of NO in the modulation of AhR-regulated genes during inflammation.

During the course of this research project, we also observed that tBHQ, a phenolic antioxidant, causes the induction of Cyp1a1 mRNA expression. Therefore, we hypothesized that tBHQ acts as an AhR ligand. To test this hypothesis, the effect of tBHQ on Cyp1a1 mRNA, protein, and enzyme activity via the XRE-dependent mechanism was investigated.

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PART II

PRESENTATION OF MANUSCRIPTS

5. DOWN-REGULATION of ARYL HYDROCARBON RECEPTOR-REGULATED GENES by TUMOR NECROSIS FACTOR-α and LIPOPOLYSACCHARIDE in MURINE HEPATOMA HEPA 1C1C7 CELLS

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated basic helix-loop-helix transcription factor (bHLH) that controls the expression of a host of different genes whose functions are linked to the metabolism of dietary constituents, drugs and potentially hazardous agents such as environmental contaminants (Burbach et al. 1992; Ema et al. 1992). The AhR exists as cytoplasmic aggregates bound to two 90-kDa heat-shock proteins (HSP90), the cochaperone p23 and the 43-kDa protein termed hepatitis B virus X-associated protein (Carver and Bradfield 1997; Ma and Whitlock 1997; Meyer et al. 1998). Upon ligand binding, the AhR dissociates from HSP90 and the ligand-receptor complex translocates to the nucleus. Then, the activated AhR dimerizes with the AhR nuclear translocator protein (ARNT), and binds to a class of promoter DNA sequences called xenobiotic responsive element (XRE) of the target genes to activate their transcription (Nebert et al. 1993; Whitlock 1999).

The AhR-regulated genes code for four phase I enzymes, cytochrome P4501A1 (CYP1A1), CYP1A2, CYP1B1, and CYP2S1 and four Phase II xenobiotic metabolizing enzymes, including NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione S-transferase A1 (GSTA1), cytosolic aldehyde dehydrogenase-3 (ALDH-3), and UDP-glucuronosyltransferase 1A6 (UGT1A6) (Nebert and Duffy 1997; Rivera et al. 2002). These enzymes are abundant in tissues that play an important role in first-pass metabolism, digestion, and drug metabolism, such as those of the gastrointestinal tract and the liver. For the most part, these enzymes could be considered to play an important role in drug metabolism. However, the induction of certain heme-thiolate proteins, such

as CYP1A1, are considered to be potentially counter productive to this process since CYP1A1 is capable of producing epoxides and dihydrodiol epoxides from aromatic and halogenated hydrocarbons (Schrenk 1998). These metabolites have been shown to be involved in the mediation of a broad range of distinct toxic responses such as immune suppression, endocrine disruption, birth defects, and carcinogenesis (Poland and Knutson 1982). The mechanism for these AhR-mediated pathophysiological conditions is not well understood.

In animals, lipopolysaccharide (LPS) or turpentine administration has been shown to down-regulate CYP1A1 and 1A2 (Bleau et al. 2000; Kurdi et al. 1999). In human, LPS increased serum concentrations of tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6 and in turn, decreased the clearance of antipyrine, hexobarbital, and theophylline (Shedlofsky et al. 1994). *In vitro*, IL-6 repressed CYP1A1, CYP1A2, and CYP3A mRNA in human hepatoma cells (Fukuda et al. 1992). IL-1β, IL-6, and TNF-α inhibited CYP1A2, CYP2D, CYP2E1, and CYP3A mRNA and related enzyme activities in human primary hepatocytes (Abdel-Razzak et al. 1993). Furthermore, inhibition of polycyclic aromatic hydrocarbon (PAH)-mediated induction of CYP1 expression by cytokines and growth factors has also been demonstrated in different cell systems *in vitro*. For example, PAH-induction of CYP1A mRNA expression is inhibited by IL-1β, IL-6, and TNF-α (Barker et al. 1992; Fukuda and Sassa 1994; Muntane-Relat et al. 1995).

Although much is known concerning the effects of inflammation on the CYP1A1, very little is known about the other AhR-regulated genes such as GSTA1 and NQO1.

However, it is well known that GSTA1 and NQO1 are highly inducible by AhR agonists such as β-naphthoflavone (βNF) or by phenolic antioxidants such as tert-butylhydroquinone (tBHQ) (Nguyen et al. 2003). βNF is also termed a bifunctional inducer, which induces both Phase I and II enzymes through AhR- and antioxidant response element (ARE)-dependent mechanisms. However, tBHQ and similar inducers are designated as monofunctional inducers, which induce only Phase II enzymes, including GSTA1 and NQO1, by activation of ARE and through AhR-independent mechanisms (Prochaska and Talalay 1988; Talalay et al. 1988). Because the levels of Phase I and II drug metabolizing enzymes in an organism may determine the potential for either metabolic activation of drugs or decreased efficacy of therapeutic molecules during inflammation, it is important to study the mechanism involved in the modulation of these enzymes during inflammation and oxidative stress. The major aim of this study was to investigate the effect of the proinflammatory cytokine, TNF-α, and LPS on the basal and inducible expression of the AhR-regulated genes Cyp1a1, Gsta1, and Nqo1 in the Hepa 1c1c7 cells.

Materials and methods

Tumor necrosis factor-α was obtained from Peprotech Canada (Ottawa, ON). Lipopolysaccharide, β-naphthoflavone, nicotinamide adenine dinucleotide phosphate, cumene hydroperoxide, dicoumarol, 2,6-dichlorophenolindophenol, flavin adenine dinucleotide, glutathione reductase, reduced glutathione, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 7-ethoxyresorufin, 7-methoxyresorufin, bovine serum albumin, Dulbecco's modified Eagle's medium base, fluorescamine, glucose, protease

inhibitor cocktail, and anti-goat IgG peroxidase secondary antibody and tert-butylhydroquinone were purchased from Sigma-Aldrich (St. Louis, MI). Tris hydrochloride, agarose, and sodium azide were purchased from EM Science (Gibbstown, NJ). Tween-20 was from BDH Inc. (Toronto, ON). Resorufin, 100X Vitamin supplement solution, and amphotericin B were purchased from ICN Biochemicals Canada (Toronto, ON). Hybond-N-nylon filters were from Amersham Canada (Oakville, ON). Penicillin/streptomycin, gentamicin, L-glutamine, fetal bovine serum, non-essential amino acid solution, TRIzol reagent and the random primers DNA labelling system were obtained from Invitrogen Canada (Burlington, ON). [α-^{32}P]dCTP (3000 Ci/mmol) was supplied by Perkin-Elmer (Boston, MA). Bromophenol blue, β-mercaptoethanol, glycine, acrylamide, *N'*, *N'*-bis-methylene-acrylamide, ammonium persulphate, nitrocellulose membrane (0.45 μm) and TEMED were purchased from Bio-Rad Laboratories (Hercules, CA). CYP1A1 goat anti-mouse polyclonal primary antibody (G-18) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Skimmed milk was obtained from DIFCO Laboratories (Detroit, MI). All other chemicals were purchased from Fisher Scientific (Toronto, ON).

Cell culture and treatments

Phenol red, which has a structural resemblance to some nonsteroidal estrogens, shows significant estrogenic activity at the concentrations (15-45 μM) which is used in cell culture media and can bind to both estrogen receptor and AhR (Berthois et al. 1986). In the present study, in order to prevent the interference of phenol red with our experiments, murine hepatoma cell lines (WT, C12, and C4 generously provided by Dr.

O. Hankinson, University of California, Los Angeles) were maintained in Dulbecco's Modified Eagle's Medium, without phenol red, supplemented with 10% fetal bovine serum, 20 μM L-glutamine, 50 μg/ml gentamicin sulfate, 100 IU/ml penicillin, 10 μg/ml streptomycin and 25 ng/ml amphotericin B. Cells were grown in 75-cm² rectangular canted neck cell culture flask with vent cap at 37°C in a 4% CO_2 humidified environment for 3 days and in order to keep the pH of media at 7.4 every other day the media were replaced with the fresh one. TNF-α was initially reconstituted with 0.1% bovine serum albumin and made up to the desired final concentration with sterile phosphate buffered-saline, pH 7.4. LPS was dissolved in sterile phosphate buffered-saline and βNF was dissolved in dimethylsulfoxide.

The cells were seeded at 7.5 x 10^4 cells/well in 96-well plates and at a cell density of 1-1.5 x 10^6 cells/well in 6-well culture plates in a Dulbecco's Modified Eagle's Medium culture media. After plating, at 24 hr (96-well plates) or 48 hr (6-well plates), the cultured cells were treated with TNF-α (1, 5 and 10 ng/ml) or LPS (1 and 5 μg/ml) in a total volume of 200 μl (96-well plates) or 2 ml (6-well plates). These cells were incubated at 37°C under a 4% CO_2 humidified environment for 6 and 24 hr for mRNA and enzymatic activity assays, respectively.

Effect of TNF-α and LPS on cell viability

Initial experiments were undertaken to determine the toxicity of TNF-α (1, 5 and 10 ng/ml) and LPS (1 and 5 μg/ml) on WT, C12 and C4 cells. Cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

(MTT) assay as described previously (Vakharia et al. 2001). The assay measures the conversion of MTT to formazan crystal by enzymes in the mitochondria of metabolically active cells. Briefly, WT, C12 and C4 cells were seeded into 96-well microtiter cell culture plates and incubated for 24 hr at 37°C in a 4% CO_2 humidified incubator. Cells were treated with varying concentrations of TNF-α (1, 5 and 10 ng/ml) or LPS (1 and 5 μg/ml). The cells were further incubated for 24 hr and the medium was again removed and replaced with cell culture medium containing 1.2 mM of MTT dissolved in phosphate buffered-saline (pH 7.2). After 2 hr of incubation, the formed crystals were dissolved with isopropanol. The intensity of the color in each well was measured at a wavelength of 550 nm using a BIO-TEK EL 312e microplate reader. TNF-α and LPS were not toxic to WT, C12 and C4 cells, at all concentrations tested (data not shown).

Determination of Cyp1a1 activity

The Cyp1a1 activity was determined by measuring the rate of *O*-deethylation of 7-ethoxyresorufin (EROD) and 7-methoxyresorufin (MROD) to resorufin (Figure 5.1).

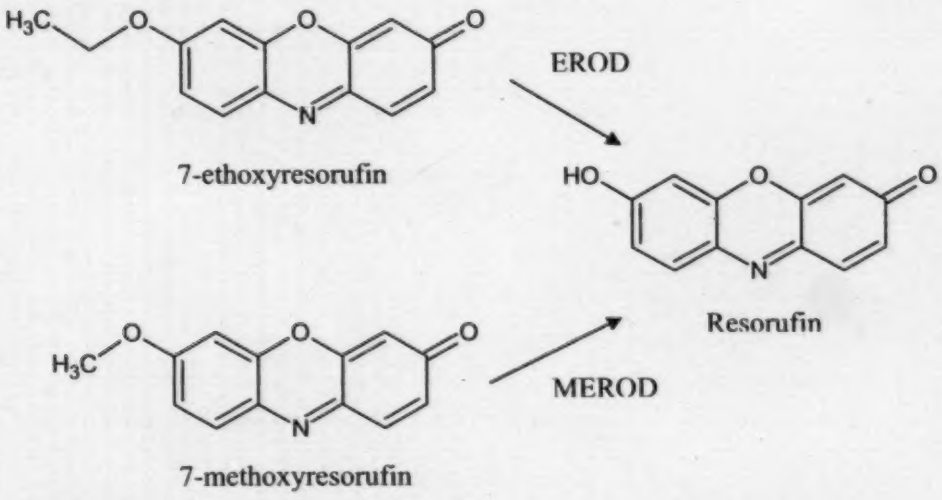

Figure 5.1. Formation of resorufin form 7-ethoxyresorufin and 7-methoxyresorufin.

EROD and MROD assays were performed on intact cells as described previously (Sinal and Bend 1997). An ethoxyresorufin or methoxyresorufin working solution was produced by dilution of ethoxyresorufin or methoxyresorufin stock solution (0.4 mM in dimethylsulfoxide) to a final concentration of 2 μM in culture medium that had been pre-warmed to 37°C. After 24 hr culture the cells in 96 sterile culture well plates, they were incubated with recombinant murine TNF-α (1, 5 and 10 ng/ ml) or LPS (1 and 5 μg/ml) in the absence or presence of β-naphthoflavone (βNF, 10 μM) for 24 hr. After aspiration of the medium containing the test compounds, 200 μl of ethoxyresorufin or methoxyresorufin working solution were then added to each cell well, and the plates were

placed in an incubator at 37°C. Series of fluorescence measurement (excitation, 545 nm; emission, 575 nm; Baxter 96-well fluorometer) were recorded from each cell well at fixed time intervals. The amount of resorufin formed in each well between the initial and final fluorescence measurements was determined by comparison with a standard curve of known concentrations. Under these assay conditions, the formation of resorufin with respect to time was verified to proceed linearly for a minimum of 15 min after the initial fluorescence measurements. To normalize resorufin values with protein concentrations, the working solution was first aspirated. The cell wells were rinsed twice with 150 µl of phosphate buffered-saline and 50 µl of distilled water were then added to lyse the cells. After placing the cell plates at -80°C for at least 1 hr, the cell lysates were allowed to thaw, and protein levels were measured as described previously (Lorenzen and Kennedy 1993). Briefly, 100 µl of phosphate buffer (pH 8.0) and 50 µl of 1 mM fluorescamine, dissolved in acetonitrile, were added to each cell well. After at least 2 minutes of shaking, the plate was scanned with a fluorescence analyzer at an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Bovine serum albumin was used to standardize the protein concentration.

Preparation of cell lysates for enzyme activity assays

24 hr after incubating the cells with the treatments, cultured cells were washed with phosphate buffered-saline. Then 0.5 ml of homogenization buffer (50 mM potassium phosphate, 1.15% KCl, pH 7.4) was added to each well of a 6-well plate. The plates were frozen for 24 hr in -80°C freezer. Thawed cells were homogenized with a Kontes homogenizer and then centrifuged at 5000 x *g* for 20 min. The supernatant

fractions were then removed and protein concentrations in the supernatants were determined by the method of Lowry et al. using bovine serum albumin as the standard (Lowry et al. 1951). The cell lysates were stored in a -20°C freezer until they were used in the determination of Gsta1 and Nqo1 enzyme activities.

Determination of Gsta1 activity

Spectrophotometric assays for Gsta1 catalytic activity using cumene hydroperoxide as a substrate were conducted according to the method of Lawrence and Burk (Lawrence 1976). These reactions were performed in 0.1 M phosphate buffered-saline (pH 6.5) at 25°C in a total volume of 1 ml. The rate of the enzyme-catalyzed reaction was determined by subtracting the rate of reaction occurring in the absence of cell homogenate. Cell homogenate protein (0.02 mg) was incubated with cumene hydroperoxide (1.5 mM), reduced glutathione (1 mM), nicotinamide adenine dinucleotide phosphate (NADPH) (0.1 mM) and glutathione reductase (0.3 unit), and the reaction was monitored for 2 min at 340 nm with $\varepsilon = 6.2\ mM^{-1}\ cm^{-1}$.

Determination of Nqo1 activity

Nqo1 activity was determined by the continuous spectrophotometric assay of Ernster et al., which quantitates the dicoumarol-inhibitable reduction of 2,6-dichlorophenolindophenol (Ernster 1967). The rate of reduction of dichlorophenolindophenol (40 μM) by NADPH (200 μM) in 1 ml of Tris-HCl buffer (pH 7.8, 25 mM) containing 0.1% Tween-20 and 0.023% bovine serum albumin was monitored for 2 min at 600 nm with $\varepsilon = 2.1\ mM^{-1}\ cm^{-1}$.

RNA extraction and Northern blot analysis

After incubation with the test compounds for the specified time periods, total cell RNA was isolated from the cells using TRIzol reagent, according to manufacturer's instructions (Invitrogen), and quantified by measuring the absorbance at 260 nm. Northern blot analysis of total RNA was performed as described elsewhere (Sambrook 1989). Briefly, aliquots of 20 µg of RNA were separated in a denaturing (0.22 M formaldehyde) agarose (1.1%) gel and transferred to Hybond-N nylon membranes. The RNA was cross-linked to the membranes using the UV Stratalinker 2400, followed by baking at 65°C for 2 hr. Prehybridization of the membranes was carried out in a solution containing: 6 x SSPE (0.9 M NaCl, 0.2 M $NaH_2PO_4.H_2O$, 0.02 M EDTA); 50% deionized formamide; 5 x Denhardt's reagent (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin); 0.5% sodium dodecyl sulfate (SDS); and 100 µg/mL sheared salmon sperm DNA, for at least 4 hr at 42°C. Hybridization with the ^{32}P-labeled cDNA probes was carried out in the same solution, minus Denhardt's reagent, for 16-24 hr at 42°C. The membranes were then washed twice at room temperature in a solution containing 2 x SSPE and 0.5% SDS for 5 min, the first wash, and 15 min for the second wash. This was followed by a 30 min wash in 0.1 x SSPE, 0.5% SDS at 42°C and a final 30 min wash in 0.1 x SSPE, 0.5% SDS at a temperature of 62°C for 30 min. The washed membranes were rinsed in a 0.1 x SSPE solution, dried, sealed in plastic wrap and visualized by exposure to Kodak Biomax MS film. Hybridization signals were quantified relative to the signals obtained for glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA.

The cDNA probes for mouse Cyp1a1 and Gapdh mRNA were generously provided by Dr. John R. Bend (University of Western Ontario, London, ON). The cDNA probe for mouse Gsta1 mRNA was provided by Dr. David Eaton (University of Washington, Seattle, WA). The mouse Nqo1 cDNA probe was prepared from previously described primer sequences by reverse transcription coupled to PCR amplification using the primers FNAD(P)H:Nqo1 5'-GCCATGGCGGCAGAAGAGCCCTG-3' (F1; forward) and BNAD(P)H:Nqo1 5'-CTTATTTTCTAGCTTTGATCTG-3' (R1; reverse) containing Nco 1 and Hind III restriction sites, respectively (Chen et al. 1994). Briefly, 5 μg of total RNA from the liver of a βNF-treated mouse was used as a template for first-strand cDNA synthesis using reverse transcriptase according to the manufacturer's (BRL) specification and by using R1 as the primer. Then, 10% of the cDNA products were added to a standard PCR mixture according to the manufacturer's specifications (BRL) using F1 and R1 as specific primers. PCR amplification was performed with a Perkin-Elmer Cetus (model 480; Norwalk, CT) DNA thermal cycler using the following temperature profile: initial denaturation for 5 min at 95°C; a step-down temperature regime where the annealing temperature is decreased each cycle (65 to 61°C) and consists of 45 sec at 95°C (denaturation), 45 sec at 65°C (annealing), 1.25 sec at 72°C (extension); followed by 35 cycles of 45 sec at 95°C (denaturation), 45 sec at 60°C (annealing), 1.25 sec at 72°C (extension), and a final extension of 8 min at 72°C. Subsequently, the 828 bp cDNA product, corresponding to nucleotides 205-1033 of the published sequence (30) Chen *et al.,* 1994), was purified from the PCR reaction by agarose (1%) gel electrophoresis and sequenced. All probes were ^{32}P-labeled by the random primer method according to the manufacturer's (Invitrogen) instructions.

Measurement of Intracellular ROS Production

Hydrogen peroxide (H_2O_2) production was examined by measuring the conversion of the cell permeant probe, 2', 7'-dichlorofluorescin diacetate to its fluorescent product, 2', 7'-dichlorofluorescein. WT, C12, or C4 cells, plated in 96-well plates, were grown for 24 h to 90% confluence. After adding 10 μM 2', 7'-dichlorofluorescin diacetate in Hanks' balanced salt solution (1.26 mM $CaCl_2.2H_2O$, 5mM KCl, 0.4 mM KH_2PO_4, 0.8 mM $MgSO_4.7H_2O$, 137 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM $Na_2HPO_4.7H_2O$, 5.5 mM Glucose) to each well, cells were treated with TNF-α (1, 5 and 10 ng/ ml) and LPS (1 and 5 μg/ml) then incubated for 2 hr at 37°C. To examine whether the AhR antagonist, αNF or the phenolic antioxidant, tBHQ have the ability to prevent TNF-α or LPS-induced ROS production, WT cells were treated with αNF (10 μM) (Gasiewicz and Rucci 1991) or tBHQ (100 μM) (Ma et al. 2004) for 2 and 24 hr, respectively, prior to treatment with TNF-α (1, 5 and 10 ng/ ml) or LPS (1 and 5 μg/ml) for 2 hr at 37°C. After 2 hr incubation with TNF-α or LPS, changes in fluorescence were measured immediately in a Baxter 96-well fluorometer (excitation and emission wavelengths of 485 and 530 nm, respectively).

Statistical analysis

All results are presented as mean ± SD (standard deviation). The comparison of the results from the various experimental groups and their corresponding controls was carried out by a one way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* tests. The differences were considered significant when $p<0.05$.

Results

Effect of TNF-α and LPS on Cyp1a1, Gsta1 and Nqo1 activities

The availability of mutant Hepa 1c1c7 cell lines deficient in AhR or ARNT allows the study of events in a signalling cascade that are dependent on the presence of both partners (AhR and ARNT) of a heterodimeric transcription factor. The AhR-deficient (C12) cells used in this study are not completely knocked out and contain about 10% AhR of that of WT cells; in contrast, ARNT-deficient (C4) cells express normal levels of AhR (Miller 1983). Both AhR and ARNT-deficient cells grow more slowly compared to the WT cells. AhR content influences both the growth and the differentiated state of Hepa 1c1c7 cells, and AhR exerts its effect on growth during the G_1 phase of the cell cycle (Ma and Whitlock 1996).

The mean basal EROD and MROD activities in the parent Hepa 1c1c7 (WT) cell line at 24 hr, the standard time of harvest, were 34.5 ± 12 and 23.6 ± 4.3 pmol/min/mg protein, respectively (Figures 5.1A, B). The mean basal specific activities of Gsta1 and Nqo1 in the WT cells were 24.2 ± 2.6 and 462.5 ± 27.3 nmol/min/mg protein, respectively (Figures 5.2A, B). As can be seen in Figures 5.4 and 5.5, baseline Gsta1 and Nqo1 activities were substantially lower than that in WT cells by up to 1.25 and 6.44 fold for C12 cells and 1.8 and 7.2 fold for C4 cells, respectively.

To determine the effect of TNF-α or LPS on AhR-regulated genes catalytic activities, the parent WT cell line and its C12 and C4 mutants were exposed to various concentrations of TNF-α (1, 5 and 10 ng/ ml) or LPS (1 and 5 μg/ml) concentrations in

the absence and the presence of βNF for 24 hr. In WT cells, TNF-α or LPS significantly inhibited the basal level of EROD activities, but not MROD activities, in a dose dependent manner (Figures 5.2). The reason for these effects is EROD activity generally correlates with the Cyp1a1 enzyme, whereas MROD activity generally but not specifically reflects the CYP1a2 enzyme (Shibazaki et al. 2004). With respect to Nqo1 and Gsta1, TNF-α or LPS significantly reduced Nqo1, but not Gsta1, basal activity dose-dependently (Figures 5.3).

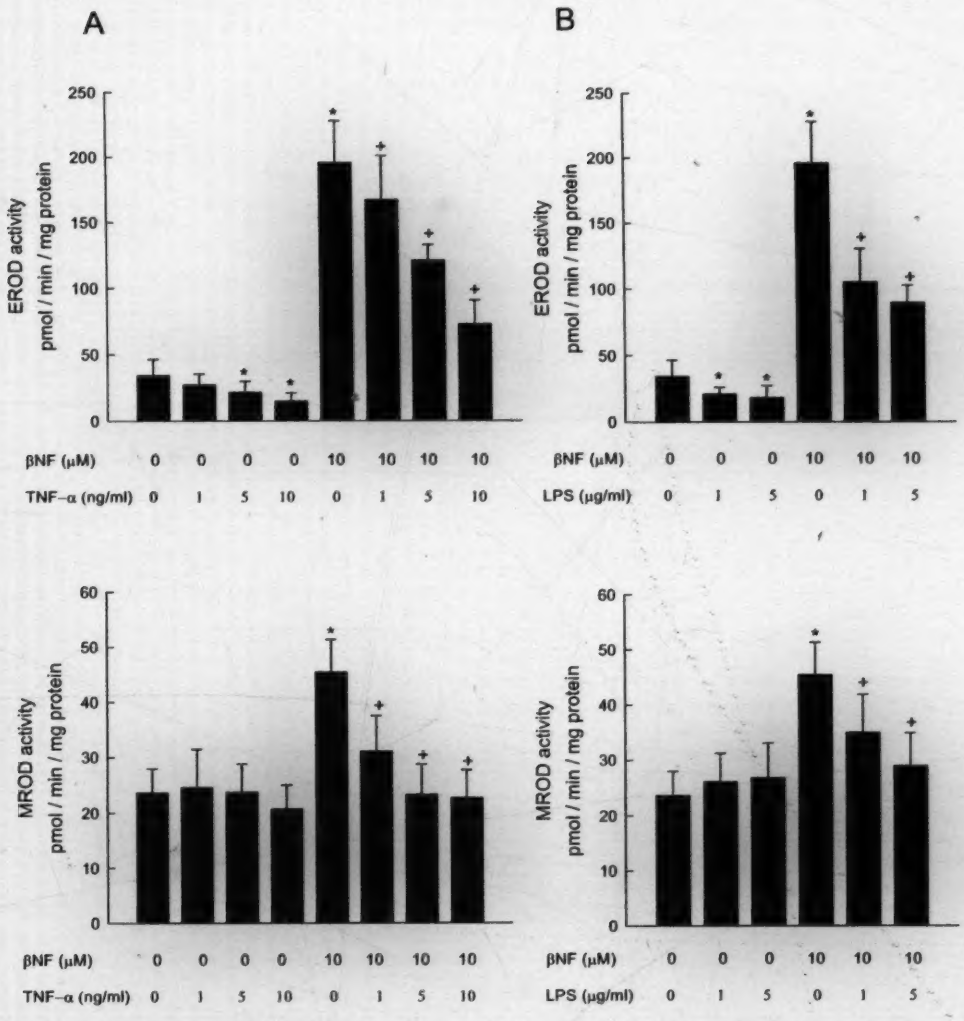

Figure 5.2. Effect of TNF-α or LPS with or without β-naphthoflavone (βNF) on EROD and MROD activities in Hepa 1c1c7 cells. Cells were treated with vehicle, recombinant TNF-α (1, 5 and 10 ng/ml), A) or LPS (1 and 5 μg/ml, B) with or without βNF (10μM) for 24 hr prior to assay. Data are expressed as mean ± SD (n = 8), $^{+}p<0.05$ compared to control, $^{*}p<0.05$ compared to βNF.

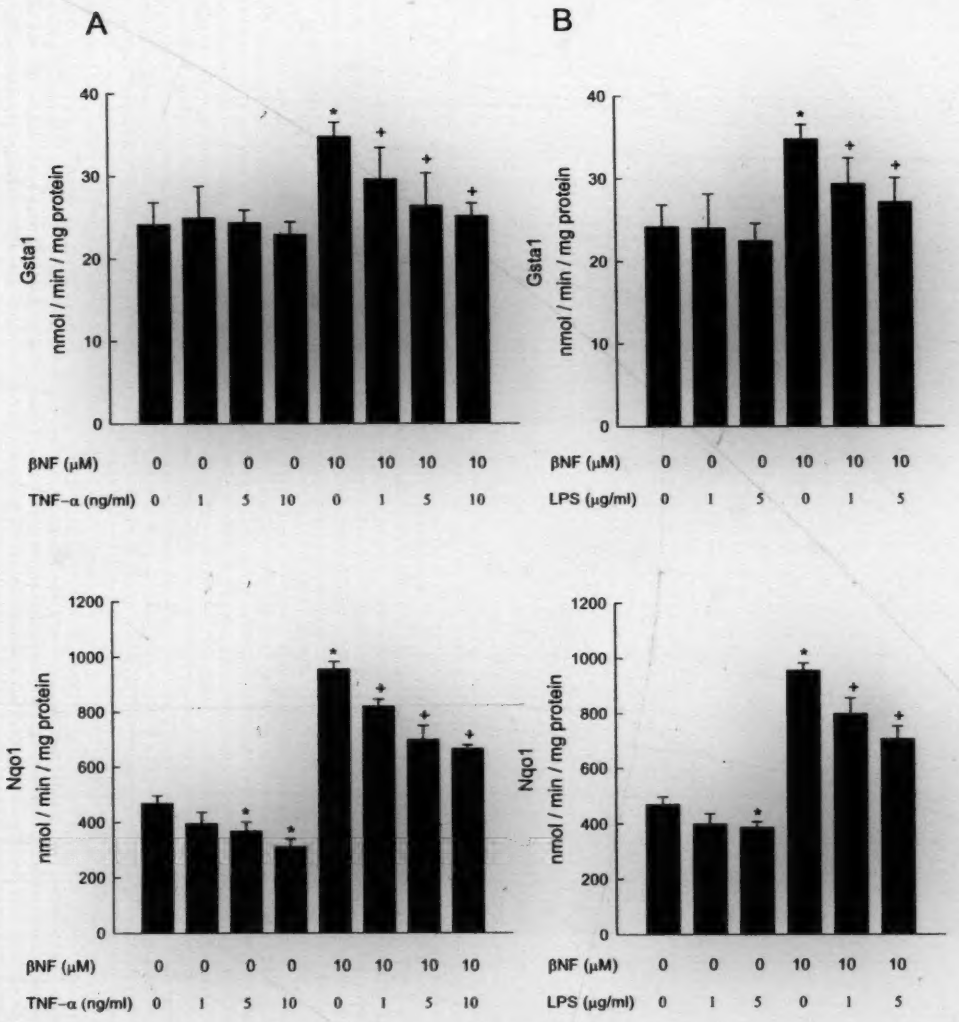

Figure 5.3. Effect of TNF-α or LPS with or without β-naphthoflavone (βNF) on Gsta1 and Nqo1 activities in Hepa 1c1c7 cells. Cells were treated with vehicle, recombinant TNF-α (1, 5 and 10 ng/ml), A) or LPS (1 and 5 μg/ml, B) with or without βNF (10μM) for 24 hr prior to assay. Data are expressed as mean ± SD (n = 8), $^{+}p<0.05$ compared to control, $^{*}p<0.05$ compared to βNF.

To verify the role of the AhR in the regulation of AhR-regulated genes and to clarify its role in modulating the effects of TNF-α or LPS, the same experiments were repeated on the AhR- and ARNT-deficient cell lines. As predicted, in both C12 and C4 the basal levels of EROD and MROD activities were non-detectable. Interestingly, in C4 cells, the basal activity of Nqo1 was induced only with LPS, although the basal level of Gsta1 activity in both C12 and C4 cells remained unchanged (Figures 5.4 and 5.5).

In WT cells, βNF caused a significant increase in the Cyp1a1, Gsta1 and Nqo1 activities by approximately 6, 1.5 and 2 fold, respectively (Figures 5.2 and 5.3). For the C12 cells, βNF only induced Nqo1 activity by 1.3 fold, but not Gsta1 activity (Figures 5.4A, B). Also as expected, no induction was observed in Gsta1or Nqo1 activities in C4 cells with βNF (Figures 5.5A, B). These data emphasize the critical role of the AhR in the regulation of Gsta1 and Nqo1.

To determine whether the TNF-α or LPS-mediated decrease in Cyp1a1, Gsta1 and Nqo1 activities is affected by an AhR ligand, the cells were treated with βNF in the absence or presence of TNF-α (1, 5 and 10 ng/ml) or LPS (1 and 5 μg/ml). When the WT cells were treated with βNF and TNF-α or βNF and LPS for 24 hr, the βNF-mediated induction of Cyp1a1, Gsta1, and Nqo1 activities were significantly inhibited in a dose-dependent manner (Figures 5.2 and 5.3) by TNF-α or LPS. In C12 cells, βNF and TNF-α or βNF and LPS did not change βNF-mediated induction of Nqo1 activities (Figures 5.4).

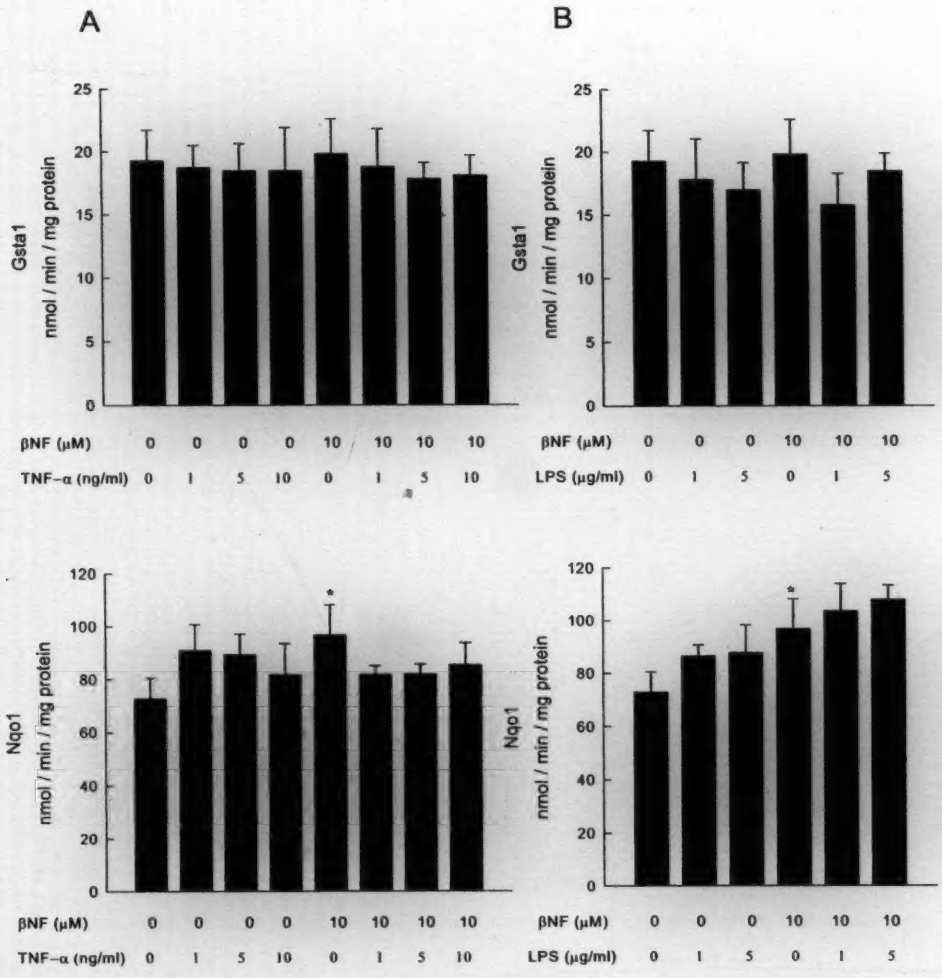

Figure 5.4. Effect of TNF-α or LPS with or without β-naphthoflavone (βNF) on Gsta1 and Nqo1 activities in Hepa 1c1c7 C12 cells. Cells were treated with vehicle, recombinant TNF-α (1, 5 and 10 ng/ml, A) or LPS (1 and 5 μg/ml, B) with or without βNF (10μM) for 24 hr prior to assay. Data are expressed as mean ± SD (n = 8), $^{+}p<0.05$ compared to control, $^{*}p<0.05$ compared to βNF.

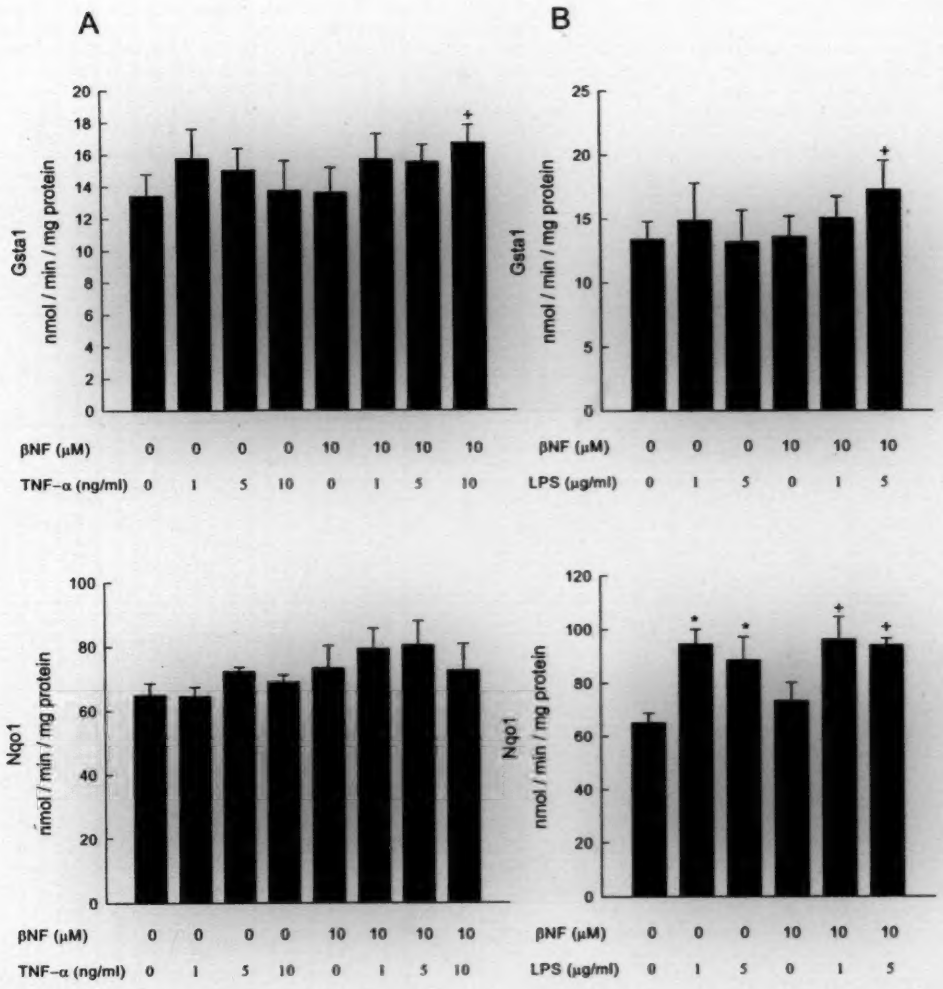
A
B
Gsta1
nmol / min / mg protein
βNF (μM) 0 0 0 0 10 10 10 10
TNF-α (ng/ml) 0 1 5 10 0 1 5 10
Gsta1
nmol / min / mg protein
βNF (μM) 0 0 0 10 10 10
LPS (μg/ml) 0 1 5 0 1 5
Nqo1
nmol / min / mg protein
βNF (μM) 0 0 0 0 10 10 10 10
TNF-α (ng/ml) 0 1 5 10 0 1 5 10
Nqo1
nmol / min / mg protein
βNF (μM) 0 0 0 10 10 10
LPS (μg/ml) 0 1 5 0 1 5

Figure 5.5. Effect of TNF-α or LPS with or without β-naphthoflavone (βNF) on Gsta1 and Nqo1 activities in Hepa 1c1c7 C4 cells. Cells were treated with vehicle, recombinant TNF-α (1, 5 and 10 ng/ml, A) or LPS (1 and 5 μg/ml, B) with or without βNF (10μM) for 24 hr prior to assay. Data are expressed as mean ± SD (n = 8), $^{+}p<0.05$ compared to control, $^{*}p<0.05$ compared to βNF.

To obtain further evidence for the involvement of AhR-dependent mechanisms, we investigated whether TNF-α or LPS could affect the induction of Gsta1 and Nqo1 by ARE. For this purpose, WT cells were treated with the phenolic antioxidant, tBHQ (100 μM), which is known to induce Gsta1 and Nqo1 through ARE, in the absence or presence of high doses of TNF-α (10 ng/ml) or LPS (5 μg/ml). As stated earlier, TNF-α or LPS alone significantly inhibited both Gsta1 and Nqo1 activities. When WT cells were incubated with tBHQ and TNF-α or tBHQ and LPS, tBHQ-mediated induction of Gsta1 and Nqo1 activities were not significantly affected by TNF-α or LPS (Figure 5.6).

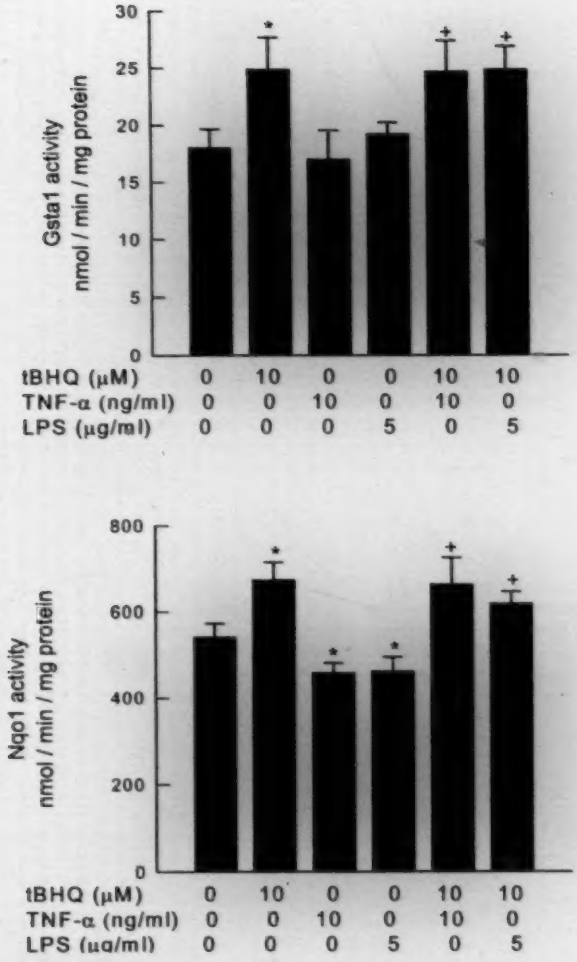

Figure 5.6. Effect of TNF-α or LPS with or without tert-butylhydroquinone (tBHQ) on Gsta1 and Nqo1 activities in Hepa 1c1c7 cells. Cells were treated with vehicle, recombinant TNF-α (10 ng/ml) or LPS (5 μg/ml) with or without tBHQ (100 μM) for 24 hr prior to assay. Data are expressed as mean ± SD (n = 6), *p<0.05 compared to control, ^{+}p<0.05 compared to TNF-α and LPS alone.

Effect of TNF-α and LPS on constitutive and inducible mRNA expression of Cyp1a1, Gsta1 and Nqo1

To determine if the modulation enzymatic activities of AhR-regulated genes were mediated at the transcriptional level, Northern blot analysis was carried out for mRNAs of Cyp1a1, Gsta1 and Nqo1. In WT cells, Cyp1a1 mRNA was not detectable in the absence of AhR ligand (βNF). Treatment of WT cells with TNF-α or LPS alone resulted in a significant decrease in the expression of Gsta1 and Nqo1 mRNA levels which paralleled the decrease in these enzyme activities (Figure 5.7). In C12 cells, TNF-α or LPS alone had no effect on the expression of Gsta1 and Nqo1 (data not shown). However, in C4 cells, LPS increased the expression of Nqo1 mRNA (data not shown), which paralleled the increase in enzyme activity.

βNF alone caused a significant increase in the mRNA expression of Cyp1a1, Gsta1 and Nqo1. The co-administration of βNF and TNF-α or βNF and LPS resulted in a dose-dependent inhibition of the βNF-mediated induction of Cyp1a1, Gsta1 and Nqo1 mRNA (Figure 5.7), which correlated with the data on enzyme activities. In C12 cells, βNF and TNF-α or βNF and LPS did not change βNF-mediated induction of Nqo1 at the activity level but caused a dose-dependent increase in Nqo1 mRNA induction by βNF. In C4 cells, βNF did not cause significant changes in activities or mRNA levels of Gsta1 or Nqo1 (data not shown).

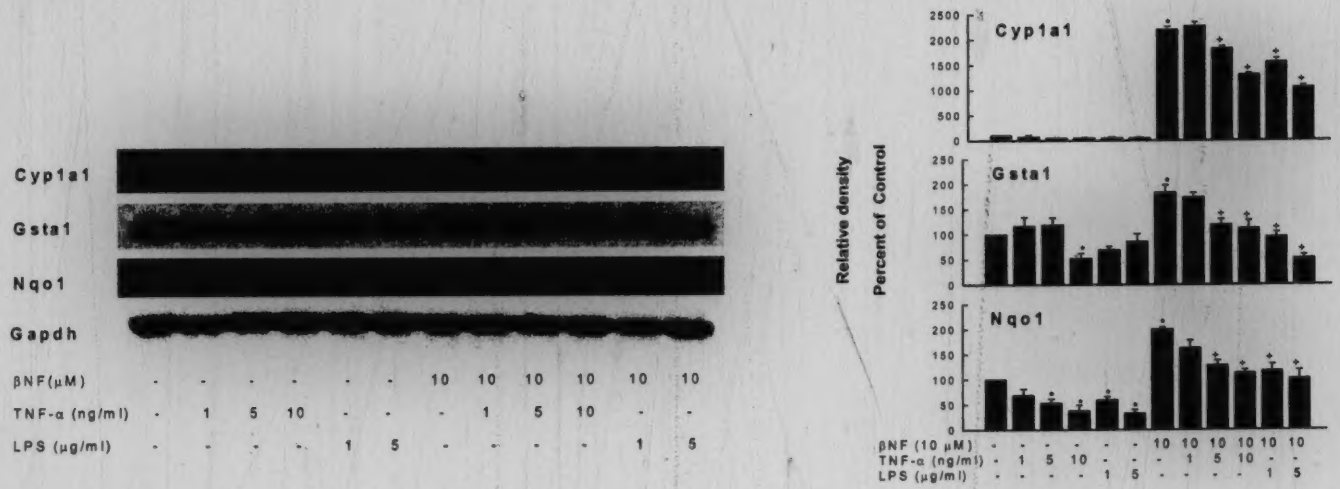
Cyp1a1
Gsta1
Nqo1
Gapdh
βNF(μM) - - - - - - 10 10 10 10 10 10
TNF-α (ng/ml) - 1 5 10 - - - 1 5 10 - -
LPS (μg/ml) - - - - 1 5 - - - - 1 5
Relative density
Percent of Control
Cyp1a1
Gsta1
Nqo1
βNF (10 μM) - - - - - - 10 10 10 10 10 10
TNF-α (ng/ml) - 1 5 10 - - - 1 5 10 - -
LPS (μg/ml) - - - - 1 5 - - - - 1 5

Figure 5.7. Effect of TNF-α or LPS with or without β-naphthoflavone (βNF) on Cyp1a1, Gsta1 and Nqo1 mRNA levels in Hepa 1c1c7 cells. Cells were treated with vehicle, recombinant TNF-α (1, 5 and 10 ng/ml) or LPS (1 and 5 μg/ml) with or without βNF (10 μM) for 6 hr prior to Northern blot analysis. The graphs represent the relative normalized amount of Cyp1a1, Gsta1 and Nqo1 mRNA (mean ± SD, $n = 3$), expressed as percentage of the control, which was normalized to Gapdh levels. $^{*}p<0.05$ compared to control, $^{+}p<0.05$ compared to βNF.

Furthermore, in accordance with the activity data, co-treatment of WT cells with tBHQ (100 μM) and high doses of TNF-α (10 ng/ml) or LPS (5 μg/ml) did not significantly affect the mRNA-induced levels of Gsta1 and Nqo1 by tBHQ (Figure 5.8).

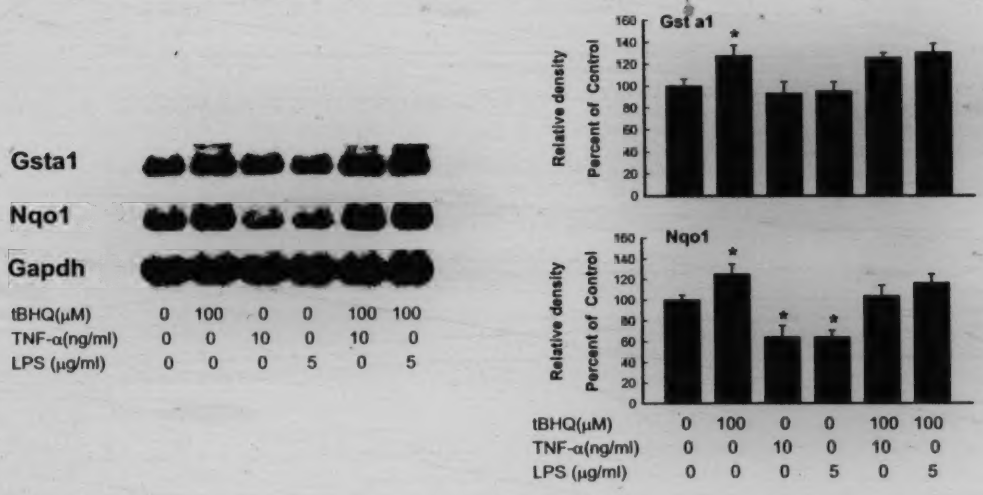
Gsta1
Nqo1
Gapdh
tBHQ(μM) 0 100 0 0 100 100
TNF-α(ng/ml) 0 0 10 0 10 0
LPS (μg/ml) 0 0 0 5 0 5
Gst a1
Relative density
Percent of Control
Nqo1
Relative density
Percent of Control
tBHQ(μM) 0 100 0 0 100 100
TNF-α(ng/ml) 0 0 10 0 10 0
LPS (μg/ml) 0 0 0 5 0 5

Figure 5.8. Effect of TNF-α or LPS with or without tert-butylhydroquinone (tBHQ) on Gsta1 and Nqo1 mRNA levels in Hepa 1c1c7 cells. Cells were treated with vehicle, recombinant TNF-α (10 ng/ml) or LPS (5 μg/ml) with or without tBHQ (100 μM) for 6 hr prior to Northern blot analysis. The graph represents the relative normalized amount of Cyp1a1 mRNA (mean ± SD, n = 3), expressed as percentage of the control, which was normalized to Gapdh levels. *p<0.05 compared to control.

Intracellular ROS production

To understand further how TNF-α or LPS alter the expression of AhR-regulated genes, we investigated whether TNF-α or LPS exposure would trigger an increase in ROS production in Hepa 1c1c7 cells. Our results clearly showed that a significant increase in ROS was observed in all three cell types (WT, C12 and C4) after exposure to TNF-α (1, 5 and 10 ng/ ml) or LPS (1 and 5 μg/ml) for 2 hr (Figure 5.9A). We also tested the effect of the AhR antagonist, αNF or the phenolic antioxidant, tBHQ on the TNF-α or LPS-induced ROS production. Our data showed that αNF did not significantly affect the TNF-α or LPS-induced ROS production, whereas tBHQ inhibited ROS production (Figures 5.9B and 9C).

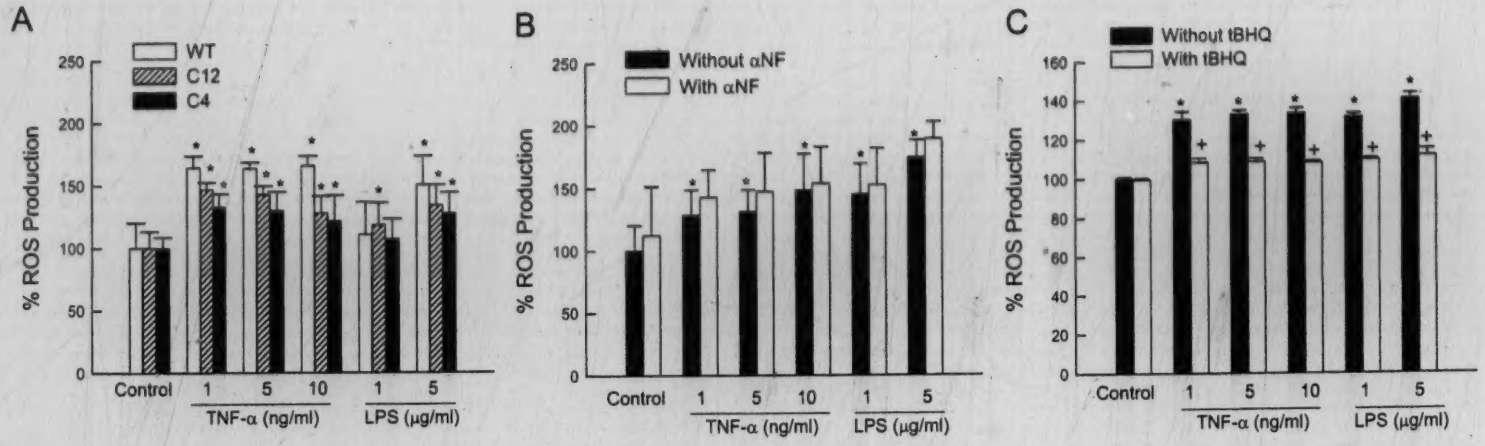

Figure 5.9. Effect of TNF-α or LPS on intracellular ROS production in the absence (A) or presence of αNF (B) or tBHQ (C) in Hepa cells. Cells were incubated with TNF-α (1, 5 and 10 ng/ml) or LPS (1 and 5 μg/ml) after pre-treatment with or without αNF (10 μM), tBHQ (100 μM) for 2 and 24 hr respectively in the presence of 2', 7'-dichlorofluorescin diacetate (5 mM). The fluorescence readings were taken after 2 hr incubation at 37°C. Data are expressed as mean ± SD (n = 12), $^{*}p<0.05$ compared to control, $^{+}p<0.05$ compared to TNF-α or LPS alone.

Discussion

In humans and animals, infections and other inflammatory stimuli cause changes in the activities and the expression levels of various drug metabolizing enzymes in the liver, as well as in extra-hepatic tissues such as lung, kidney and brain (Morgan 1997). Usually, drug metabolizing enzymes and their activities are suppressed by such stimuli, though some are unaffected or may be induced. Suppression of drug metabolizing enzymes can result in increased clinical toxicity of drugs with low therapeutic indices (Morgan 1997). Conversely, suppression of the metabolism during an inflammatory response may lead to reduced therapeutic or toxic effects of drugs that are metabolized to pharmacologically or toxicologically active compounds.

In the present study, the role of AhR in suppression of AhR-regulated genes by TNF-α or LPS is demonstrated based on the results that TNF-α or LPS repressed the βNF-mediated induction of Cyp1a1, Gsta1, and Nqo1 in WT but not in C12 and C4 cells. Further evidence for this conclusion comes from the observation that the induction of Gsta1 and Nqo1 at both activity and mRNA levels by tBHQ were not significantly affected by TNF-α or LPS. The changes at the enzyme activity levels coincided with the changes in gene expression, suggesting that a pre-transcriptional mechanism is involved in the modulation of AhR-regulated genes by TNF-α or LPS. In agreement with other studies, our results showed that Cyp1a1 mRNA was not detectable at the basal level (Nemoto and Sakurai 1992). However, we found that the basal activity of Cyp1a1, which was measured by EROD assay, was detectable and down-regulated by various concentrations of TNF-α (5 and 10 ng/ml) or LPS (1 and 5 μg/ml). The contribution of

the other enzymes such as NADPH-cytochrome P450 reductase and NQO1 in formation of resorufin from 7-ethoxyresorufin during dealkylation may be the reason of the controversy between different studies (Dutton and Parkinson 1989; McCallum et al. 1993).

Several AhR-dependent mechanisms may have contributed to the down-regulation of AhR-regulated genes by TNF-α or LPS. It is known that LPS and proinflammatory cytokines, such as TNF-α and IL-1β, activate the nuclear factor kappa B (NF-κB) (Ghosh et al. 1998). NF-κB is a pleiotropic transcription factor that participates in the regulation of many physiological responses, including inflammatory reactions, cell proliferation, immune responses, apoptosis and developmental processes (Baldwin 1996). The classic inducible NF-κB heterodimer typically consists of a p65 (RelA) and a p50 subunit, with RelA being the subunit conferring strong transcriptional activation (Baeuerle and Henkel 1994).

Of interest, it has been demonstrated that there is a mutual inhibitory interaction between the AhR and the NF-κB signalling pathways, suggesting that NF-κB may play a direct role in mediating the suppression of CYP1A1 expression by inflammatory agents (Tian et al. 1999). Furthermore, it has been noted that transcriptional coregulators such as nuclear receptor coactivators and corepressors which are involved in histone-acetylation mediate the cross-interactions between the AhR and NF-κB signalling pathways (Ke et al. 2001). Histone-acetylation of the Cyp1a1 promoter, an important initial step for gene activation, was found to be suppressed by treatment with TNF-α or LPS through

activation of NF-κB (Ke et al. 2001). It is conceivable that the cross-interactions between the AhR and NF-κB signalling pathways also converge upon these coactivators and corepressors. Competition between ligand-AhR/ARNT complexes and RelA for binding of coactivators and corepressors could affect the level of transcriptional activation seen in these two pathways. Interestingly, it has been reported that tBHQ prevents the activation of NF-κB induced by TNF-α or LPS, suggesting that the protective effect of tBHQ on suppression of AhR-regulated genes by TNF-α or LPS may be due to the inhibition of NF-κB activity (Ma and Kinneer 2002).

It has also been shown that LPS suppresses Cyp1a1 mRNA expression directly through the activation of the MAPK (ERKs, JNKs, and p38) pathway via its membrane-bound receptor, Toll-like receptor (TLR) (Guha and Mackman 2001). It was suggested that the AhR pathway and the MAPK pathway may cross-talk with each other, but the detailed mechanism remains controversial (Guha and Mackman 2001; Shibazaki et al. 2004).

It has recently been shown that ROS such as H_2O_2 suppress CYP1A1 and CYP1A2 expression (Morel and Barouki 1999; Morel et al. 1999; Takemura et al. 1999). The exact mechanism by which H_2O_2 down-regulates CYP remains unclear, but it may be caused by H_2O_2 interaction with the enzyme-associated Fe^{2+}, leading to heme destruction and enzyme inactivation (Archakov et al. 1998; Karuzina and Archakov 1994). Morel and Barouki have reported that the *CYP1A1* gene is repressed by oxidative stimuli via modulation of the binding of nuclear factor-1 (NF-1) to the *CYP1A1* promoter (Morel and

Barouki 1998). Suppression of *CYP1A1* promoter activity by TNF-α was dependent on an intact NF-1 binding site, and the antioxidant pyrrolidine dithiocarbamate inhibited the CYP1A1 down-regulation, suggesting that TNF-α regulates CYP1A1 via redox regulation of NF-1 (Morel and Barouki 1998).

In the current study, we have demonstrated that the decrease in AhR-regulated genes by TNF-α or LPS was associated with an increase in ROS production. The increase in ROS production induced by TNF-α or LPS was significantly prevented by the phenolic antioxidant, tBHQ, while it was not significantly affected by the AhR antagonist, αNF, suggesting that the protective effect of tBHQ on AhR-regulated genes is due to the inhibition of ROS production. In addition, the AhR may not be involved in the production of ROS by TNF-α or LPS. In agreement with our results it has also been reported that tBHQ inhibits TNF-α- or LPS-induced ROS production (Ma and Kinneer 2002). Interestingly, it has been previously reported that ROS can activate NF-κB activity and the increase in NF-κB activity can be prevented by antioxidants (Baeuerle and Henkel 1994; Flohe et al. 1997; Janssen-Heininger et al. 2000). These results suggest that the increase in ROS production by TNF-α and LPS plays an important role in the CYP1A1 suppression, possibly through the activation of NF-κB activity.

Another potential mediator that may be involved in the suppression of AhR-regulated genes by TNF-α or LPS is nitric oxide (NO). We have previously shown that TNF-α significantly increases NO formation in Hepa 1c1c7 cells (Gharavi and El-Kadi 2003). It is well established that substantial quantities of NO are released during

inflammation and in response to immune stimuli such as TNF-α or LPS (Carlson and Billings 1996). NO is capable of binding to the heme protein of CYP enzymes, blocking the binding of O_2 and thus inhibiting the activity of enzyme (Paton and Renton 1998). Also, in the presence of ROS, NO can form highly reactive species, including peroxynitrite, capable of oxidizing amino acids critical to the functioning of the enzyme, with resultant irreversible loss of catalytic activity (Bogdan 2001).

In conclusion, we have demonstrated that the down-regulation of AhR-regulated genes by TNF-α and LPS is AhR-dependent. In addition, ROS may be involved in the suppression of AhR-regulated genes by TNF-α and LPS.

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6. *TERT*-BUTYLHYDROQUINONE IS A NOVEL ARYL HYDROCARBON RECEPTOR LIGAND

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Introduction

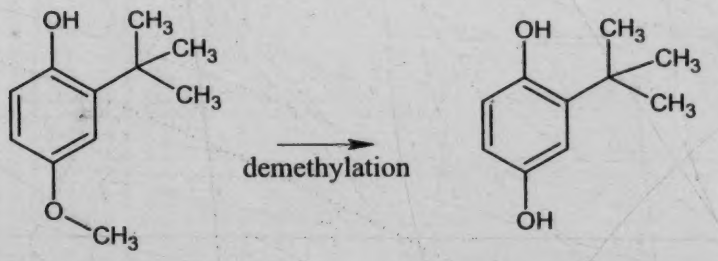
The aryl hydrocarbon receptor (AhR) is a ligand-activated basic helix-loop-helix transcription factor (bHLH) that controls the expression of different genes whose functions are linked to the metabolism of dietary constituents, drugs, and potentially hazardous agents such as environmental contaminants (Burbach et al. 1992; Ema et al. 1992). The AhR exists as cytoplasmic aggregates bound to two 90-kDa heat-shock proteins (HSP90), the cochaperone p23 and a 43-kDa protein termed hepatitis B virus X-associated protein (Carver and Bradfield 1997; Ma and Whitlock 1996; Meyer et al. 1998). Upon ligand binding, the AhR dissociates from HSP90 and the ligand-receptor complex translocates to the nucleus. Then, the activated AhR dimerizes with the AhR nuclear translocator protein (ARNT), and binds to a class of promoter DNA sequences called xenobiotic responsive elements (XRE) of the target genes to activate their transcription (Nebert et al. 1993; Whitlock 1999). The AhR-regulated genes consist of four phase I enzymes, cytochrome P450 1A1 (CYP1A1), CYP1A2, CYP1B1, and CYP2S1 and several phase II enzymes that are involved in the metabolism of xenobiotics and endogenous compounds (Nebert et al. 2000; Rivera et al. 2002).

Among the AhR-regulated genes, CYP1A1 is the most capable of producing polar, toxic or even carcinogenic metabolites from various AhR ligands, including aromatic and halogenated hydrocarbons (Schrenk 1998). These metabolites have been shown to be involved in the mediation of a broad range of distinct toxic responses such as immune suppression, endocrine disruption, birth defects, and carcinogenesis (Poland and Knuston 1982).

tert-Butylhydroquinone (tBHQ) is a major metabolite of 3-*tert*-butyl-hydroxyanisole (BHA) *in vivo* in dogs, rats, and humans (Figure 6.1) (Nakamura et al. 2003). BHA has been widely used for many years as an antioxidant to preserve and stabilize the freshness, nutritive value, flavour and colour of foods and animal food products (Li et al. 2002; Williams et al. 1999). Both tBHQ and BHA have received much attention due to their abilities to induce phase II detoxification enzymes, including glutathione S-transferase and NADPH: quinone oxidoreductase, and thus their potential role in cancer prevention (Li et al. 2002). In contrast to the beneficial effects of BHA, a number of studies have shown that chronic exposure to high concentrations of BHA in the diet induces neoplastic lesions in the forestomach, urinary bladder and esophagus of rats, mice, hamsters, and pigs (Li et al. 2002).

Although the carcinogenicity of BHA in animals, particularly at high concentrations, has been well documented, the relevance of these results to the low exposure of humans to BHA has been questioned (Li et al. 2002). Recently, a study on the carcinogenicity of BHA, at low concentrations, alone or in combination with other phenolic chemicals, including caffeic acid, sesamol, 4-methoxyphenol and catechol, in rats has shown that the above phenolic compounds exerted synergistic effects on rat forestomach carcinogenesis (Li et al. 2002). Assessing the risk from human consumption of BHA and tBHQ is complicated by the fact that the precise mechanisms of their carcinogenicity are not well understood. The metabolic formation of tBHQ has been suggested to, at least in part, contribute to the carcinogenic effect of BHA.

In an attempt to investigate the mechanisms by which tBHQ induces toxicity or carcinogenicity, we examined the effect of tBHQ on Cyp1a1 mRNA, protein and enzyme activity in murine hepatoma Hepa 1c1c7 cells. The involvement of the AhR-dependent signalling pathway was also investigated by using an AhR antagonist and gel electrophoretic mobility shift assay (EMSA). Here, we provide the first direct evidence for an AhR-dependent induction of *Cyp1a1* gene expression and enzyme activity by tBHQ, which has a single unsaturated phenolic ring (Figure 6.1)



3-*tert*-butyl-hydroxyanisole (BHA) *tert*-Butylhydroquinone (tBHQ)

Figure 6.1. Chemical structure of 3-*tert*-butyl-hydroxyanisole and *tert*-Butylhydroquinone.

Materials and Methods

Materials. *tert*-Butylhydroquinone, resorufin, cycloheximide, actinomycin D, resveratrol, bovine serum albumin, Dulbecco's modified Eagle's medium base, 7-ethoxyresorufin, glucose, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, protease inhibitor cocktail, and anti-goat IgG peroxidase secondary antibody were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Tris hydrochloride,

agarose, and sodium azide were purchased from EM Science (Gibbstown, NJ). Tween-20 was from BDH Inc. (Toronto, ON). Amphotericin B and 100X vitamin supplements were purchased from ICN Biomedicals Canada (Montreal, Quebec). Gentamicin sulfate, penicillin–streptomycin, L-glutamine, MEM non-essential amino acids solution, fetal bovine serum, TRIzol reagent, and the random primers DNA labelling system were purchased from Invitrogen Co. (Grand Island, NY). Poly(dI/dC), Hybond-N nylon membranes and chemiluminescence Western blotting detection reagents were from Amersham Canada (Oakville, ON). [α-^{32}P]dCTP and [γ-^{32}P]ATP (3000 Ci/mmol) were supplied by DNA Core Services Laboratory, University of Alberta. Bromophenol blue, β-mercaptoethanol, glycine, acrylamide, *N'N'*-bis-methylene-acrylamide, ammonium persulphate, nitrocellulose membrane (0.45 μm), and TEMED were purchased from Bio-Rad Laboratories (Hercules, CA). Cyp1a1 and ARNT goat anti-mouse polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Skim milk was obtained from DIFCO Laboratories (Detroit, MI). All other chemicals were purchased from Fisher Scientific (Toronto, ON).

Cell Culture and Treatments. Murine hepatoma Hepa 1c1c7 (generously provided by Dr. O. Hankinson, University of California, Los Angeles, CA) and human hepatoma HepG2 cells (obtained from the American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's modified Eagle's medium, without phenol red, supplemented with 10% fetal bovine serum, 20 μM L-glutamine, 50 μg/ml gentamicin sulfate, 100 IU/ml penicillin, 10 μg/ml streptomycin and 25 ng/ml amphotericin B, 0.1 mM non-essential amino acids, and vitamin supplement solution. Cells were grown in 75-cm²

rectangular canted neck cell culture flask with vent cap at 37°C in a 4% CO_2 humidified environment. Cell culture media was changed every 2 days in order to keep the pH at 7.4.

tBHQ and resveratrol were dissolved in dimethylsulfoxide. Actinomycin D (Act-D) and cycloheximide (CHX) were dissolved in 75% ethanol and sterile distilled water, respectively. For all experiments, control groups for each of the treatments were administered the appropriate solvent. For analysis of mRNA and protein expression levels, ~1×10^6 cells were added to a 6-well tissue culture plate in 2 ml of culture media. For analysis of EROD activity, ~1×10^5 cells were added to each well of a 96-well tissue culture plate in 200 µl of culture media. On 60-80% confluence (1-2 days), appropriate stock solutions of the test chemicals were directly added to the culture media. For experiments involving Act-D, resveratrol and CHX the first two chemicals were added 2 h, while CHX was added 0.5 h, before treatment of cells with tBHQ.

Effect of tBHQ on Cell Viability. The effect of tBHQ on cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Vakharia et al. 2001). The assay measures the conversion of MTT to formazan crystal by enzymes in the mitochondria of metabolically active cells. Briefly, Hepa 1c1c7 cells were seeded into 96-well microtiter cell culture plates and incubated for 24 h at 37°C in a 5% CO_2 humidified incubator. Cells were treated with various concentrations of tBHQ (0, 1, 10, 50, 100, and 500 µM). After 24 h incubation, the medium was removed and replaced with cell culture medium containing 1.2 mM of MTT dissolved in phosphate buffer saline (pH 7.4). After 2 h of incubation, the formed crystals

were dissolved with isopropanol. The intensity of the color in each well was measured at a wavelength of 550 nm using the BIO-TEK EL 312e microplate reader.

Determination of Cyp1a1 Activity. The Cyp1a1-dependent 7-ethoxyresorufin *O*-deethylase (EROD) activity was performed on intact, living cells as described previously (Elbekai and El-Kadi 2004; Korashy and El-Kadi 2005). Enzymatic activity was normalized for cellular protein content which was determined using a modified fluorescent assay (Elbekai and El-Kadi 2004; Korashy and El-Kadi 2005).

RNA Extraction and Northern Blot Analysis. After incubation with the test compounds for the specified time periods, total cellular RNA was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen), and quantified by measuring the absorbance at 260 nm. Northern blot analysis of total RNA was performed as described elsewhere (Sambrook et al. 1989). Briefly, aliquots of 20 µg of RNA were separated in a denaturing (2.2 M formaldehyde) agarose (1.1%) gel and transferred to Hybond-N nylon membranes. The RNA was cross-linked to the membranes using the UV Stratalinker 2400, followed by baking at 65°C for 2 h. Prehybridization of the membranes was carried out in a solution containing: 6 x SSPE (0.9 M NaCl, 0.2 M $NaH_2PO_4.H_2O$, 0.02 M EDTA); 50% deionized formamide; 5 x Denhardt's reagent (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin); 0.5% sodium dodecyl sulfate (SDS); and 100 µg/ml sheared salmon sperm DNA, for at least 4 h at 42°C. Hybridization with the ^{32}P-labeled cDNA probes was carried out in the same solution, minus Denhardt's reagent, for 16-24 h at 42°C. The membranes were then washed twice

at room temperature in a solution containing 2 x SSPE and 0.5% SDS for 5 min for the first wash, and 15 min for the second wash. This was followed by a 30 min wash in 0.1 x SSPE, 0.5% SDS at 42°C and a final 30 min wash in 0.1 x SSPE, 0.5% SDS at a temperature of 62°C for 30 min. The washed membranes were rinsed in a 0.1 x SSPE solution, dried, sealed in plastic wrap and visualized by exposure to Kodak Biomax MS film. The intensities of the Cyp1a1 mRNA were quantified, relative to the signals obtained for glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA using a Java-based image processing software, ImageJ [W. Rasband (2005) National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij.].

The cDNA probes for mouse Cyp1a1 and Gapdh mRNA were generously provided by Dr. John R. Bend (University of Western Ontario, London, ON). All probes were ^{32}P-labeled by the random primer method according to the manufacturer's (Invitrogen) instructions.

Western Blot Analysis. Cells treated with tBHQ for 24 h were collected in lysis buffer containing 50 mM HEPES, 0.5 M sodium chloride, 1.5 mM magnesium chloride, 1 mM EDTA, 10% (v/v) glycerol, 1% Triton X-100, and 5µl/ml of protease inhibitor cocktail. The cell lysates incubated on ice for 1 h, with intermittent vortexing every 10 min, followed by centrifugation at 12,000 *g* for 10 min at 4°C. Proteins (100 µg) were resolved by denaturing electrophoresis, as described previously (Elbekai and El-Kadi 2004; Korashy and El-Kadi 2005; Sambrook et al. 1989). Briefly, the supernatant fractions were dissolved in 1x sample buffer, boiled for 5 min, separated by 7.5% SDS-PAGE bis-

acrylamide gel electrophoresis and electrophoretically transferred to a nitrocellulose membrane. Protein blots were blocked for 24 h at 4°C in blocking buffer containing 5% skim milk powder, 2% bovine serum albumin and 0.05% (v/v) Tween-20 in tris-buffered saline solution (TBS; 0.15 M sodium chloride, 3 mM potassium chloride, 25 mM Tris-base). After blocking, the blots were incubated with a primary polyclonal goat anti-mouse Cyp1a1 antibody for 2 h at room temperature in TBS containing 0.05% (v/v) Tween-20 and 0.02% sodium azide. Incubation with a peroxidase-conjugated rabbit anti-goat IgG secondary antibody was carried out in blocking buffer for 1 h at room temperature. The bands were visualized with the enhanced chemiluminescence method according to manufacturer's instructions (Amersham, Arlington Heights, IL).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis of mRNA. RT was performed using Murine Leukemia Virus reverse transcriptase (Invitrogen) as described previously with slight modification (Li et al. 1998). Briefly, 2.5 μg of purified total RNA was used in 40 μL RT reaction containing 8 μM oligo (dT), 1 mM dNTP, 40 U RNase, 10 mM dithiothreitol, and 400 U reverse transcriptase (Invitrogen). The reaction was incubated at 37° for 10 min and 42° for 1 h, then heated to 70° for 15 min to inactivate the RT. 2.5 μl of RT reaction mixture was subjected to PCR amplification with 0.96 μM specific forward and reverse primers for CYP1A1, (0.2 μM for β-Actin) in a 50 μL reaction containing 0.25 mM dNTPs, 1.25 U Taq polymerase (Invitrogen), and 2 mM $MgCl_2$. PCR was performed using AmpliTAQ DNA Polymerase (Perkin–Elmer) according to the manufacturer's instructions. cDNA was denatured at 94° for 4 min and cycled immediately 30 times at different temperatures including, 94° for 20 sec.

(denaturation), 49° for CYP1A1 and 52° for β-Actin for 20 sec (annealing), and 72° for 40 sec (extension). The PCR reaction ended with 7 min incubation at 72°. β-Actin was used as the internal standard to normalize for RNA loading and PCR variation. 1.5% agarose gel electrophoresis was used to separate PCR-amplified products. Ethidium bromide was added to the gel before solidification. PCR products were visualized under a UV transluminator and digitally recorded. Band intensity was quantitated using a Java-based image processing software, ImageJ [W. Rasband (2005) National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij.]. Primer sequences used were: CYP1A1 forward, 5'-GACCTGAATGAGAAGTTCTACAGC-3'; reverse, 5'-CGGAAGGTCTCCAGGATGAAG-3' and β-actin forward, 5'-CTACAATGAGCTGCGTGTGG-3'; reverse, 5'-TAGCTCTTCTCCAGGGAGGA-3' as described previously (Li et al. 1998; Roblin et al. 2004).

Nuclear Extracts and EMSA. Nuclear extracts were prepared as described previously (Rogers and Denison 2002). Briefly, cells treated for 1 h at 37°C with the indicated chemicals or solvent were first washed with PBS and incubated with 10 mM Hepes buffer for 15 min. Then cells were pelleted and homogenized in 1 ml of MDH buffer containing 3 mM $MgCl_2$, 1 mM DTT, and 25 mM HEPES (pH 7.5) per plate. The homogenates were then centrifuged at 1,000*g* for 5 min at 4°C. The pellet was washed twice by resuspending it in 1 ml of MDHK (MDH + 0.1 M KCl) per plate and recentrifuged at 1,000*g* for 2 min at 4°C. The washed pellet was resuspended in 25 μl of HDKG containing 25 mM HEPES (pH 7.5), 1 mM DTT, 0.4 M KCl, and 10% glycerol (v/v) per plate and incubated for 20 min on ice with vortexing every 5 min. The incubated

pellet was centrifuged at 12,000*g* for 20 min at 4°C. The resulting supernatant was centrifuged at 100,000*g* for 1 h at 4°C to obtain the final nuclear extract. A complementary pair of synthetic oligonucleotides containing the XRE3 binding site for the transformed AhR/ARNT complex (5'-GATCTGGCTCTTCTCACGCAACTCCG-3'and 5'-GATCCGGAGTTGCGTGAGAAGAGCCA-3') were synthesized, purified, and annealed by heating to 90°C for 5 min, then allowed to cool to room temperature (Denison et al. 1988). The double-stranded XRE was then labeled with [γ^{32}P]ATP at the 5-end using T4 polynucleotide kinase (Invitrogen), according to the manufacturer's instructions, and used as a probe for EMSA reactions. For EMSA analysis, 10 µg of nuclear extract proteins were incubated for 30 min at room temperature in a reaction mixture containing 1.8 µg of poly (dI/dC), 25 mM Hepes (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol (v/v), and 80 mM KCl, followed by incubation for another 30 min with ~1 ng (100,000 c.p.m.) [^{32}P]XRE. After incubation, the reaction mixture was then separated by electrophoresis through a 4% nondenaturing polyacrylamide gel. The gel was dried at 80°C for 1h, and then visualized by autoradiography. For competition experiments, nuclear extracts were preincubated at room temperature for 30 min with a 100-fold molar excess of unlabelled XRE or 1 µg of anti-ARNT antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) before the addition of the labelled XRE. Preincubation with antibody made to the C-terminal region of ARNT is known to inhibit AhR/ARNT heterodimer binding to XRE (Santiago-Josefat et al. 2001).

Statistical Analysis. All results are presented as mean ± SD (standard deviation). The comparison of the results from the various experimental groups and their corresponding

controls was carried out by a one way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* tests. The differences were considered significant when $p<0.05$.

Results

Effect of tBHQ on Cell Viability. To determine the optimal concentrations to use in our studies, tBHQ was tested for potential cytotoxicity in Hepa 1c1c7 cells. Figure 6.2 shows that tBHQ at concentrations of 1-100 μM did not affect cell viability. However, 500 μM, the highest concentration tested, caused a 59% decrease in the cell viability. Therefore, all subsequent studies were conducted using concentrations of 1-100 μM.

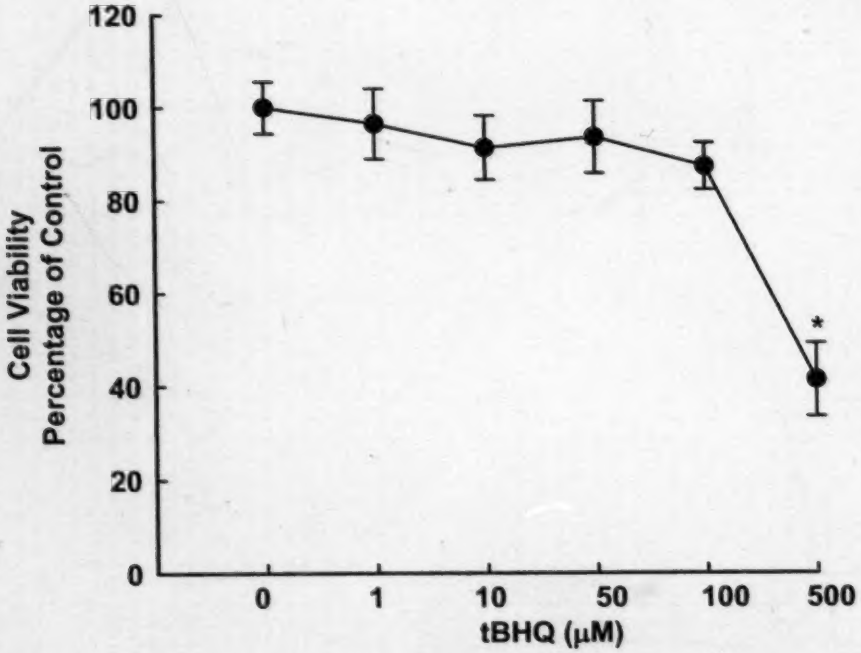


Figure 6.2. Effect of tBHQ on cell viability. The cell viability was tested 24 h after treatment of Hepa 1c1c7 cells with tBHQ (0, 1, 10, 50, 100, and 500 μM), by measuring the conversion of MTT to formazan crystals. Data are expressed as percent of untreated control, which is set at 100%, ± SD (n = 8).

Increase in Cyp1a1 Expression after tBHQ Treatment. Northern blot analysis was performed to examine the time-dependent effect of tBHQ on Cyp1a1 mRNA. At a concentration of 100 μM, tBHQ caused a time-dependent increase in Cyp1a1 mRNA level (Figure 6.3). The onset of induction was achieved only 3 h after the addition of tBHQ, but Cyp1a1 mRNA levels remained elevated for at least 12 h post tBHQ treatment.

To examine the concentration-dependent effect of tBHQ on Cyp1a1 mRNA, the cells were treated with various concentrations of tBHQ (1-100 μM). Figure 6.4 illustrates that tBHQ caused a concentration-dependent increase in Cyp1a1 mRNA levels already apparent at a concentration of 10 μM, whereas maximum induction was obtained at about 100 μM.

To assess whether the increase in Cyp1a1 mRNA level was accompanied by an increase in protein expression, quantitative measurement of Cyp1a1 protein using Western blot analysis was carried out. Our results clearly show that only the highest concentrations tested, 50 and 100 μM of tBHQ caused significant induction in Cyp1a1 protein levels (Figure 6.5).

Furthermore, to assess the functional implication of exposure to tBHQ, Cyp1a1-dependent EROD activity was also measured in Hepa 1c1c7 cells (Figure 6.6). Significant increases in EROD activity were measured at 10, 50 and 100 μM of tBHQ (1.6, 3.3 and 3.0 fold increase compared to control, respectively), which correlates with the mRNA data.

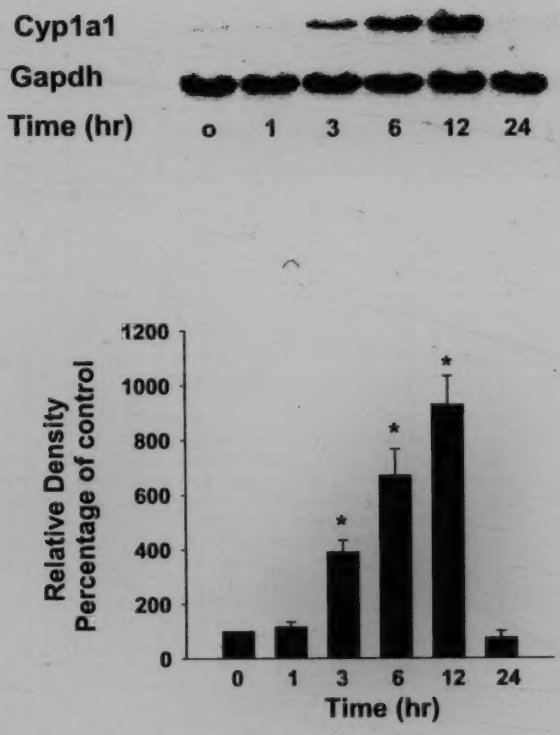


Figure 6.3. Time-dependent increase in Cyp1a1 mRNA, 0, 1, 3, 6, 12, and 24 h after treatment of Hepa 1c1c7 cells with tBHQ (100 μM), as assessed by Northern blot analysis. The graph represents the relative normalized amount of Cyp1a1 mRNA (mean ± SD, n = 3), expressed as percentage of the control, which was quantified by ImageJ software and normalized to GAPDH levels. One of three representative experiments is shown. * $p<0.05$ compared to control.

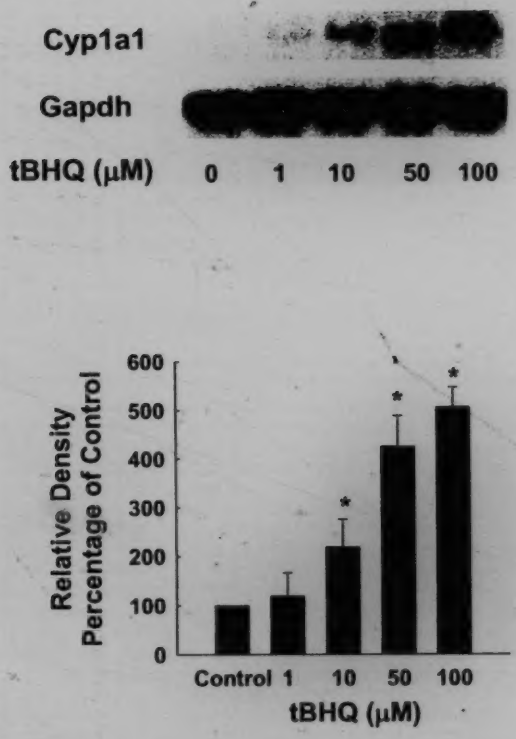


Figure 6.4. Concentration-dependent increase in Cyp1a1 mRNA, 6 h after treatment of Hepa 1c1c7 cells with tBHQ (0, 1, 10, 50, and 100 μM), as assessed by Northern blot analysis. The graph represents the relative normalized amount of Cyp1a1 mRNA (mean ± SD, n = 3), expressed as percentage of the control, which was quantified by ImageJ software and normalized to GAPDH levels. One of three representative experiments is shown. $^{*}p<0.05$ compared to control.

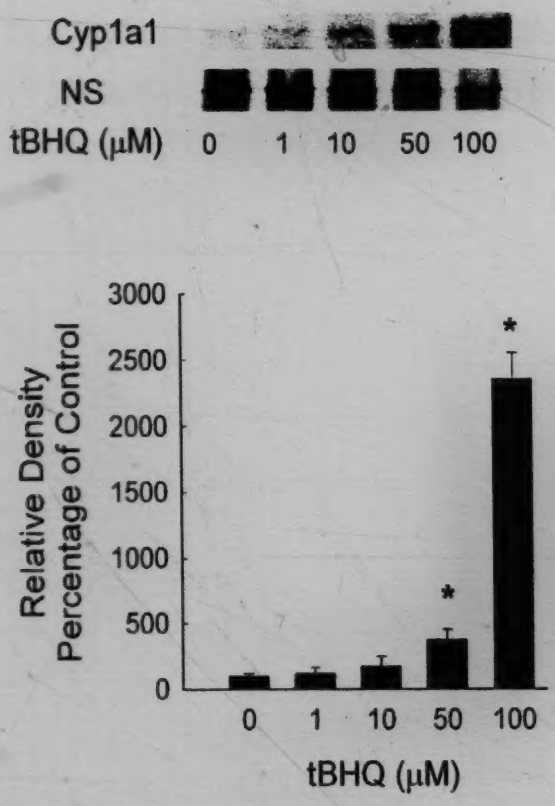


Figure 6.5. Concentration-dependent increase in Cyp1a1 protein, 24 h after treatment of Hepa 1c1c7 cells with vehicle or tBHQ (0, 1, 10, 50, and 100 μM), as assessed by Western blot analysis. The graph represents the relative normalized amount of Cyp1a1 protein (mean ± SD, $n = 3$), expressed as percentage of the control, which was quantified by ImageJ software and normalized to nonspecific band, NS, from the same membrane used as a loading control. One of three representative experiments is shown. $^{*}p<0.05$ compared to control.

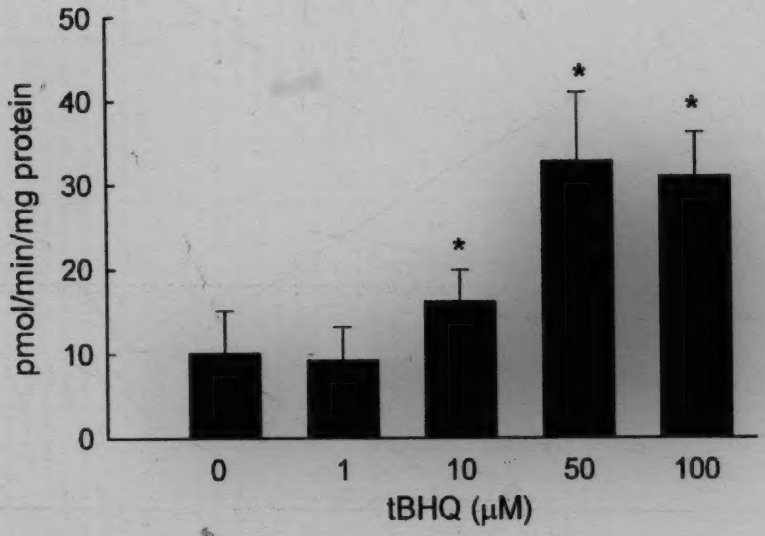


Figure 6.6. Concentration-dependent increase in Cyp1a1-dependent EROD activity, 24 h after treatment of Hepa 1c1c7 cells with tBHQ. Cells were treated with vehicle or tBHQ (0, 1, 10, 50, and 100 μM) for 24 h prior to assay. Data are expressed as mean ± SD (n = 8), $^{*}p<0.05$ compared to control.

Effect of tBHQ on CYP1A1 in HepG2 cells. To examine whether tBHQ causes similar effects in human cells, human hepatoma HepG2 cells were treated with various concentrations of tBHQ (0-100 μM). Similar to the results obtained with Hepa 1c1c7 cells, tBHQ caused a concentration-dependent increase in CYP1A1 at the mRNA and activity levels in HepG2 cells (Figure 6.7A and B). However, at the highest concentration tested, 100 μM, the induction in CYP1A1 mRNA and activity was less than that obtained with the 50 μM concentration, probably due to reduced cell viability (about 25% decrease in the cell viability (Figure 6.8).

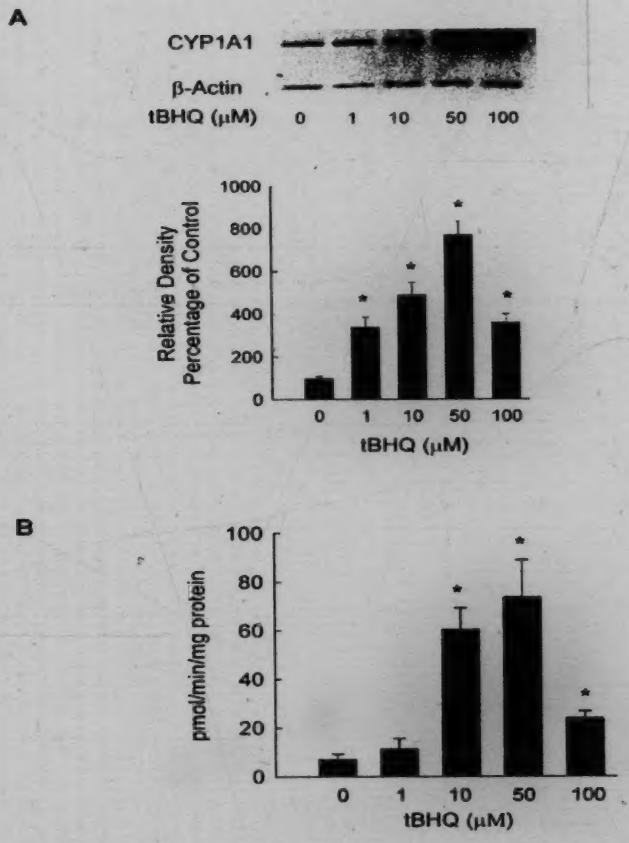


Figure 6.7. Concentration-dependent increase in Cyp1a1 mRNA and activity levels in HepG2 cells. Cells were treated with vehicle or tBHQ (0, 1, 10, 50, and 100 μM) for 6 and 24 h for mRNA and EROD activity, respectively. A, Cyp1a1 mRNA was determined using the RT-PCR method as described under *Materials and Methods*. The graph represents the relative normalized amount of Cyp1a1 protein (mean ± SD, n = 3), expressed as percentage of the control, which was quantified by ImageJ software and normalized to β-Actin as internal control B, Cyp1a1-dependent EROD activity was determined as described under *Materials and Methods*. $^{*}p<0.05$ compared to control.

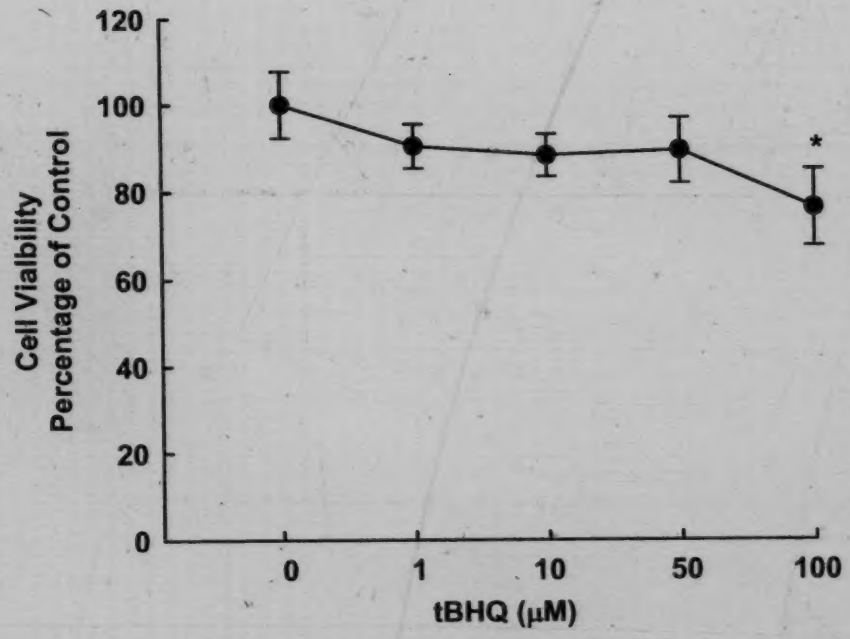


Figure 6.8. Effect of tBHQ on Hep G2 cell viability. The cell viability was tested 24 h after treatment of HepG2 cells with tBHQ (0, 1, 10, 50, and 100 μM), by measuring the conversion of MTT to formazan crystals. Data are expressed as percent of untreated control, which is set at 100%, ± SD ($n = 8$).

Effect of RNA and Protein Synthesis Inhibitors. To determine whether the increase in Cyp1a1 expression in response to tBHQ was a result of *de novo* RNA synthesis or a post-transcriptional effect, Hepa 1c1c7 cells were co-treated with the RNA polymerase inhibitor Act-D. Treatment with 5 μg/ml Act-D (Lee and Safe 2001) 2 h before exposure to 100 μM tBHQ completely abolished the increase in Cyp1a1 mRNA (Figure 6.9A). Act-D also completely blocked the increase in Cyp1a1-dependent EROD activity in response to 100 μM tBHQ (Figure 6.9B).

We also determined whether the increased cyp1a1 mRNA levels due to tBHQ exposure were dependent on the action of a highly labile or *de novo* synthesized protein. Hepa 1c1c7 cells were treated with the protein synthesis inhibitor, CHX (1 μg/ml) (Sinal and Bend 1997) in the presence or absence of tBHQ (100 μM) for 6 h. Our results clearly demonstrated that in the absence of tBHQ, CHX alone significantly increased the Cyp1a1 mRNA level. In addition, co-treatment of cells with CHX and tBHQ superinduced the increase in Cyp1a1 mRNA levels by CHX (Figure 6.10A). With respect to EROD activity, CHX significantly inhibited the Cyp1a1 induction in response to tBHQ treatment (Figure 6.10B), indicating a requirement for *de novo* protein synthesis for increased EROD activity.

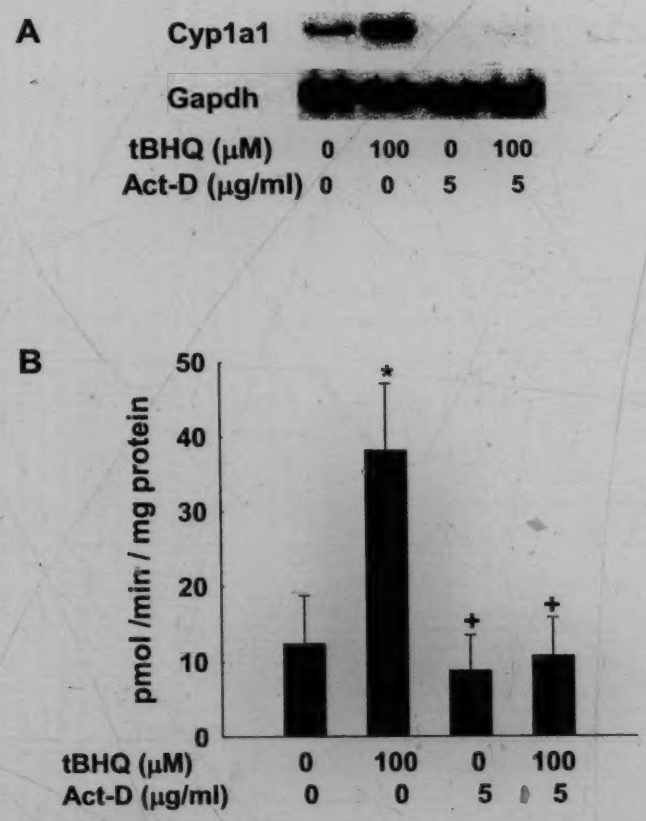


Figure 6.9. A, Inhibition of tBHQ-mediated increase in Cyp1a1 mRNA in Hepa 1c1c7 cells by Act-D, as assessed by Northern blot analysis. Cells were pretreated with Act-D (5 μg/ml) 2 h before exposure to tBHQ (100 μM) for a subsequent 6 h time period. The graph represents the relative normalized amount of Cyp1a1 mRNA (mean ± SD, $n = 3$), expressed as percentage of the control, which was quantified by ImageJ software and normalized to GAPDH levels. B, Inhibition of tBHQ-mediated induction of Cyp1a1-dependent EROD activity in Hepa 1c1c7 cells by Act-D. Cells were treated with vehicle or 100 μM tBHQ with or without Act-D (5 μg/ml) for 24 h prior to assay. $^{*}p<0.05$ compared to control, $^{+}p<0.05$ compared to tBHQ.

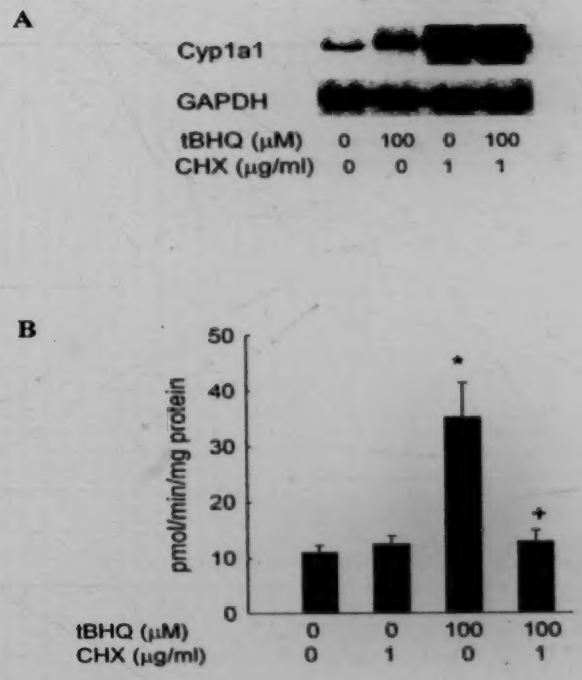


Figure 6.10. A, Effect of CHX on tBHQ-mediated increase in Cyp1a1 mRNA in Hepa 1c1c7 cells as assessed by Northern blot analysis. Cells were pretreated with CHX (1 μg/ml) 0.5 h before exposure to tBHQ (100 μM) for a subsequent 6 h time period. The graph represents the relative normalized amount of Cyp1a1 mRNA (mean ± SD, $n = 3$), expressed as percentage of the control, which was quantified by ImageJ software and normalized to GAPDH levels. B, Inhibition of tBHQ-mediated the induction of Cyp1a1-dependent EROD activity in Hepa 1c1c7 cells by CHX. Cells were treated with vehicle or tBHQ (100 μM) with or without CHX (1 μg/ml) for 24 h prior to assay. $^{*}p<0.05$ compared to control, $^{+}p<0.05$ compared to tBHQ.

AhR-Dependent Induction of Cyp1a1. To investigate further the role of AhR in the induction of Cyp1a1 by tBHQ, Hepa 1c1c7 cells were incubated with an AhR antagonist, resveratrol (100 μM) (Andrieux et al. 2004) 2 h prior to treatment with tBHQ (100 μM). Our results demonstrated that resveratrol significantly inhibited the increase in Cyp1a1-dependent EROD activity in response to tBHQ (Figure 6.11). The contribution of the other enzymes such as NADPH-cytochrome P450 reductase and NQO1 in formation of resorufin from 7-ethoxyresorufin during dealkylation may explain the lack of effect of resveratrol on the constitutive expression of Cyp1a1 in the absence of tBHQ (Dutton and Parkinson 1989; McCallum et al. 1993)

Ligand-dependent activation of the AhR/ARNT heterodimer to a DNA-binding form (XRE), so called transformation, can be monitored by incubation of nuclear proteins with the ligand *in vitro* followed by EMSA. EMSA results (Figure 6.12), demonstrate that tBHQ significantly induces the formation of a heterodimer/^{32}P-XRE complex that comigrates with that induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The specificity of tBHQ-induced AhR/ARNT heterodimer binding to XRE was confirmed by competition experiments in the presence of 100-fold molar excess of unlabeled XRE or by the addition of anti-ARNT antibody.

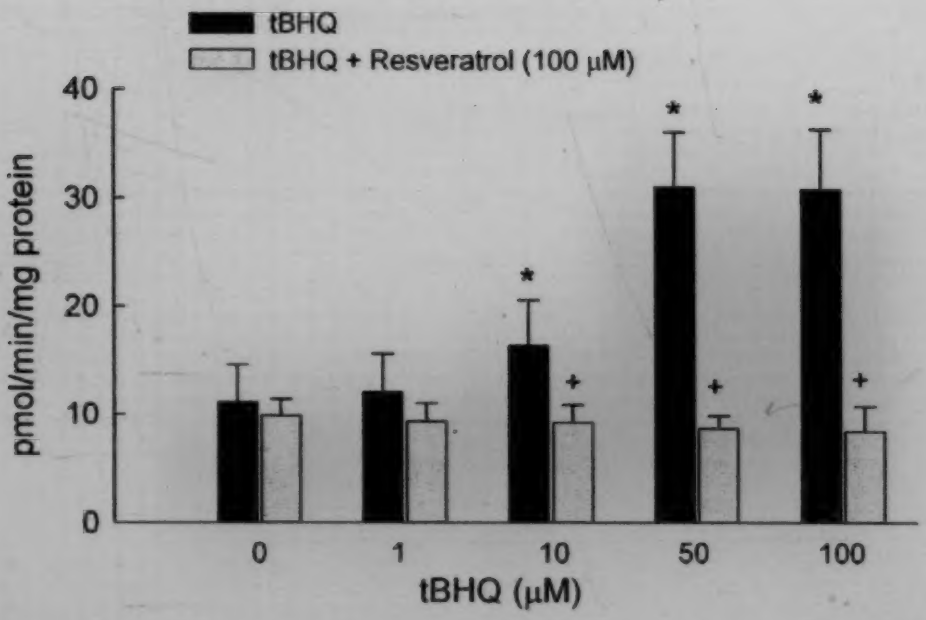


Figure 6.11. Inhibition of concentration-dependent increase in Cyp1a1-dependent EROD activity in Hepa 1c1c7 cells by resveratrol. Cells were treated with vehicle or tBHQ (0, 1, 10, 50, and 100 μM) with or without resveratrol (100 μM) for 24 h prior to assay. Data are expressed as mean ± SD (n = 8), $^{*}p<0.05$ compared to control, $^{+}p<0.05$ compared to tBHQ.

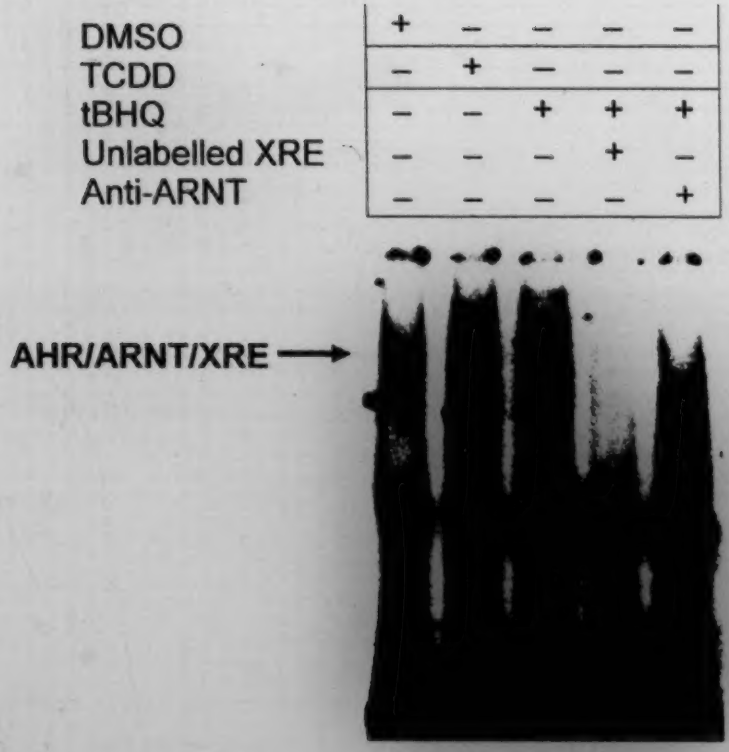


Figure 6.12. Formation of XRE-binding complexes from Hepa 1c1c7 cell nuclear extracts after tBHQ treatment. Cells were treated with vehicle or tBHQ (100 μM) or TCDD (10 nM) for 2 h. Nuclear extracts from these treated cells were incubated with a ^{32}P-labeled XRE oligonucleotide, and the formation of protein/DNA complexes was analyzed by EMSA (n = 3). The *arrow* indicates the specific binding of the AhR/ARNT complex to XRE. The specificity of the shifted band was confirmed by preincubating nuclear extracts from Hepa 1c1c7 cells with a 100-fold molar excess of unlabeled XRE or with 1 μg of anti-ARNT antibody for 30 min before the addition of the labelled XRE.

Discussion

To date, numerous chemicals have been identified as AhR ligands (Denison et al., 2002). Most of them, the "classical" ligands, including polycyclic aromatic hydrocarbons (PAHs) and halogenated aryl hydrocarbons, share the structural features of being planar, aromatic and hydrophobic (Denison and Nagy 2003). Recently, a relatively large number of AhR ligands whose structures and physiochemical characteristics differ from classical ligands have been identified (Denison and Nagy 2003). The majority of these "non-classical" AhR ligands have a low affinity to the AhR and are relatively weak inducers of Cyp1a1, compared to TCDD. A wide range of structural diversity in AhR ligands indicates that a greater spectrum of chemicals can interact with and activate this receptor than previously thought (Denison and Nagy 2003). Here, our data provide strong evidence that tBHQ, which has a single unsaturated phenolic ring, is a weak ligand of AhR compared to TCDD, and can directly induce *Cyp1a1* gene expression and enzymatic activity in an AhR-dependent manner.

Humans consume appreciable amounts of phenolic antioxidants, including BHA and its active metabolite, tBHQ, as food additives from dietary sources (Ma and Kinneer 2002). tBHQ has been found to be a prototype inducer of a non-receptor signalling pathway which selectively induces phase II enzymes in an AhR-independent manner and exhibits anticarcinogenic effects (Li et al. 2002; Munzel et al. 2003). Induction of phase II enzymes by tBHQ requires an antioxidant responsive element (ARE) located in the enhancers of genes and is mediated through an Nrf2-dependent signal transduction (Ma and Kinneer 2002). Nrf2 is a redox-sensitive member of the CNC bzip (cap 'n' collar

basic leucine zipper) family of transcription factors that forms a cytoplasmic complex with Keap1 (Ma and Kinneer 2002). In the presence of tBHQ, Nrf2 dissociates from Keap1 and translocates into the nucleus, followed by dimerization with Maf (musculoaponeurotic fibrosarcoma) protein, binding to ARE, and subsequently inducing transcription of genes (Ma and Kinneer 2002). Studies on Nrf2-null mice revealed that lack of expression of Nrf2 markedly enhances the susceptibility of the mice to cancer by benzo[a]pyrene. Thus induction of phase II enzymes through Nrf2 can account for chemoprotection by tBHQ against certain carcinogens (Ma and Kinneer 2002). On the other hand, evidence is being provided that the bioactivation of tBHQ through a Cu(II)/Cu(I) redox cycle mechanism results in the formation of 2-tert-butyl(1,4)paraquinone (tBQ), semiquinone anion radical and reactive oxygen species (ROS). The ROS may be involved in oxidative DNA damage and hence contribute to the carcinogenicity of BHA (Li et al. 2002). However, other mechanisms may also be involved.

Despite the extensive interest in the effect of tBHQ on human health, little is known about the physiologically relevant concentration attainable in human plasma and tissue, but it has been estimated that the daily consumption of tBHQ may reach 28-42 mg/day (European Food Safety Authority 2004). Moreover, it has been reported that when a single dose of 125 mg tBHQ was given orally to adult male volunteers, the serum level of tBHQ was 31-37 mg/l, which is equivalent to 187-223 μM (European Food Safety Authority 2004). Considering the daily consumption of tBHQ is about 28-42 mg we estimate the plasma concentration will range from 50-75 μM.

To understand further the mechanisms involved in BHA carcinogenesis, we hypothesized that tBHQ acts as an AhR ligand and induces Cyp1a1. The reason is that the conversion of AhR ligands into electrophilic compounds by Cyp1a1 results in the formation of covalent adducts which can react directly with intracellular nucleophiles, including DNA, and initiate the cancer process (Spink et al. 2002). To test this hypothesis, we investigated the effect of tBHQ on Cyp1a1 mRNA expression, protein and EROD activity in Hepa 1c1c7 cells. Our data clearly show that treatment of Hepa 1c1c7 cells with tBHQ (10-100 μM) causes concentration-dependent induction in Cyp1a1 mRNA and EROD activity. However, at the protein level, only the highest concentrations tested, 50 and 100 μM, caused significant induction in Cyp1a1 protein levels. Similarly, in human HepG2 cells, tBHQ caused significant induction of CYP1A1 at the mRNA and activity levels. In agreement with our study, Sugatani et al. have shown that treatment of HepG2 cells with tBHQ at 80 μM for 24 h produced a significant induction of CYP1A1 mRNA, while other studies have reported that treatment of Hepa 1c1c7 cells with 25 μM or 500 μM of tBHQ had no effect on Cyp1a1 at the mRNA or activity levels (Lamb and Franklin 2002; Liu et al. 1994; Sugatani et al. 2004; Vasiliou et al. 1995). We have found that tBHQ does not induce Cyp1a1 expression at 500 μM due to increased cytotoxicity at that concentration (Fig. 6.2).

Other aspects of our data are also worth noting. The time course study for Cyp1a1 mRNA induction indicates that the increase in response to tBHQ was somewhat delayed, with the increase in Cyp1a1 mRNA levels being clearly achieved 3 h post tBHQ treatment. However, the onset of the Cyp1a1 mRNA increase was rapid and readily

apparent by 1 h for TCDD (Korashy and El-Kadi 2005). In addition, induction of EROD activity 24 h after treatment with 100 μM tBHQ was only 40% of that observed for TCDD. Taken together, these data suggest that tBHQ induces Cyp1a1, albeit with lower affinity than TCDD.

Further evidence for the involvement of the AhR comes from the result of the EMSA. Presence of a nuclear AhR complex is dependent on ligand binding by the cytosolic receptor, nuclear translocation of the liganded AhR, its heterodimerization with ARNT and subsequent specific and high-affinity DNA binding of the heteromeric transformed AhR complex within the nucleus (Phelan et al. 1998). Our results not only suggest that tBHQ can induce AhR transformation, nuclear accumulation, and DNA binding, similar to that observed with other AhR ligands such as TCDD, but also support a role for the AhR in the induction of Cyp1a1. To support our conclusion, the EROD activity induced by tBHQ was significantly prevented by the AhR antagonist, resveratrol, suggesting a direct contribution of an AhR-dependent mechanism.

We also attribute the changes in mRNA expression to a direct effect on transcription. Inhibition of tBHQ-mediated induction of Cyp1a1 mRNA in Hepa 1c1c7 cells treated with the RNA synthesis inhibitor Act-D demonstrates a requirement for *de novo* mRNA synthesis, consistent with increased gene transcription mediated by the ligand-bound AhR complex. It has been previously reported that in Hepa 1c1c7 cells, superinduction of Cyp1a1 mRNA is a transcriptional event involving a liganded AhR (Israel et al. 1985) and is considered to reflect the existence of a labile repressor protein

that inhibits the response of the receptor-enhancer system through protein-protein interactions (Lusska et al. 1992). Our results demonstrate that CHX alone significantly increased the Cyp1a1 mRNA, which was superinduced in the presence of CHX and tBHQ, indicating that the superinduction requires the activation of AhR by an agonist. Therefore, tBHQ increases the *Cyp1a1* gene expression through an AhR-dependent mechanism. The Cyp1a1-mediated induction by CHX alone, in the absence of an additional exogenous inducing agent, has been noted in various cell types (Giachelli et al. 1991). In addition, inhibition of tBHQ-mediated induction of Cyp1a1 activity by CHX indicates that *de novo* Cyp1a1 protein synthesis is required.

Taken together, the results provided here present us with the first evidence that tBHQ, a phenolic antioxidant, can directly modulate the expression of Cyp1a1 through an AhR-dependent pathway by acting as an AhR ligand. In addition, induction of Cyp1a1 by tBHQ may be directly or indirectly involved in BHA carcinogenesis.

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7. MEASUREMENT of NITRIC OXIDE METABOLITE (NITRITE) in MURINE HEPATOMA HEPA1C1C7 CELLS BY REVERSED PHASE HPLC with FLUORESCENCE DETECTION

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Introduction

Nitric oxide (NO), an unstable molecule, is biosynthesized from the amino acid L-arginine by at least three different isoforms of NO synthase (neuronal, NOS1; inducible, NOS2; endothelial, NOS3) (Southan and Szabo 1996). Activation of different cell types such as macrophages, endothelial cells, fibroblasts and hepatocytes with pro-inflammatory cytokines results in the expression of a distinct isoform of NO synthase (iNOS) (Xie et al. 1992). The resulting NO free radical species may quickly react with molecular oxygen and water to form a variety of end products, including nitrite (NO_2^-), nitrate (NO_3^-), and S-nitrosothiols (RSNO) (Moncada et al. 1991; Nathan 1992).

NO is produced by various cell types in the picomolar to nanomolar range and has a very short half-life ($t_{1/2}$ <5s) in biological fluids; therefore, a direct measurement of its production is difficult and the analysis of NO_2^- and NO_3^-, the stable products of NO oxidation, is often performed to estimate NO levels in biological fluids and cell culture medium (Moncada et al. 1991).

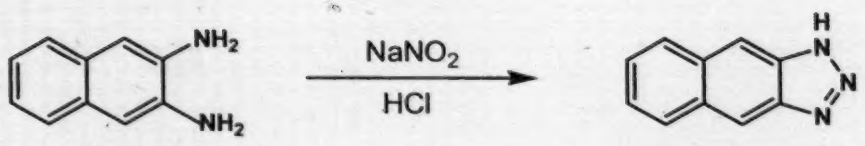
Several methods of measuring NO_2^- such as the Griess colorimetric assay (Egami et al. 1974; Nagano 1999), chemiluminescence analysis (Cox 1980), gas chromatographic analysis (Johnson and Burleson 1996), hemoglobin trapping (Nagano 1999) and a fluorometric method (Misko et al. 1993) have been developed. However, the most commonly employed method for the analysis of NO_2^- in aqueous solutions is the Griess colorimetric assay (Egami et al. 1974; Nagano 1999). The Griess assay is a well-known two-step diazotization reaction in which NO_2^- in the sample reacts with sulfanilic acid to produce the diazonium ion, which is then coupled to N-(naphthyl)ethylenediamine to

form the chromophormic azo derivative (Miles et al. 1996). Although the Griess reaction is simple and fast, its detection limit for NO_2^- is only 0.1 – 1 μM (Nagano 1999). The lack of sensitivity restricts the application of this colorimetric method for quantifying micromolar levels of NO_2^- and NO_3^- in biological samples (Li et al. 2000). On the other hand, the chemiluminescence assay is considered the most useful method for highly sensitive real-time assay of unstable radicals such as NO at low concentrations in physiological solutions and its detection limit is approximately 100 fM (Nagano 1999). The chemiluminescence assay, however, requires an expensive and bulky apparatus, and there is also an interference between NO and N-nitro-L-arginine (a commonly used inhibitor of NO synthesis) and some nitroso compounds such as S-nitrosothiols and nitrosodiphenylamine (Cox 1980; Hampl et al. 1996). The oxyhemoglobin (HbO_2) oxidation method or hemoglobin trapping is based on the oxidation reaction of HbO_2 to methemoglobin (MetHb) by NO (Nagano 1999). However, there are two problems with this method. First, it is difficult to reproducibly prepare a constant amount of HbO_2. The second problem is that HbO_2 reacts with NO_2^- to form MetHb. Therefore, it is intrinsically difficult to differentiate NO from its decomposed product (Nagano 1999).

Besides the above mentioned methods, a number of ion exchange (El Menyawi et al. 1998; Everett et al. 1995; Jedlickova et al. 2002; Marzinzig et al. 1997; Meyer et al. 1997; Miles et al. 1996; Nussler et al. 2002; Preik-Steinhoff and Kelm 1996; Smith et al. 2002; Stratford et al. 1997), reversed phase ion-paired (Michigami et al. 1989; Stein et al. 1988) and reversed phase (Kumarathasan et al. 2001; Li et al. 2000; Woitzik et al. 2001) HPLC methods have been developed for measuring NO_2^- and NO_3^- in biological systems using UV-VIS absorbance (El Menyawi et al. 1998; Marzinzig et al. 1997;

Michigami et al. 1989; Smith et al. 2002; Stein et al. 1988), conductivity (Stratford et al. 1997), electrochemical (Jedlickova et al. 2002; Preik-Steinhoff and Kelm 1996) or fluorescence detectors (Kumarathasan et al. 2001; Li et al. 2000; Meyer et al. 1997; Woitzik et al. 2001). Most HPLC methods require several purification steps to remove interfering substances such as biogenic amines (El Menyawi et al. 1998; Kumarathasan et al. 2001; Smith et al. 2002) and need prior filtration before injection of the samples to the HPLC system. These additional preparative steps may cause variable recovery and introduce contamination by environmental NO_2^- and NO_3^- (Li et al. 2000). In addition, the HPLC methods with UV-VIS, conductimetric, electrochemical or some fluorescence detection suffer from low sensitivity (El Menyawi et al. 1998; Marzinzig et al. 1997; Meyer et al. 1997; Nussler et al. 2002; Stratford et al. 1997) compared with the fluorometric method and chemiluminescence assays (Cox 1980; Hampl et al. 1996; Misko et al. 1993; Zafiriou and McFarland 1980).

The batch fluorometric assay is based on the reaction of NO_2^- with 2,3-diaminonaphthalene (DAN), as an indicator of NO formation, under acidic conditions to yield the fluorescent product 2,3-naphthotriazole (NAT) (Figure 7.1) (Wiersma 1970). The minimum detectable amount of NO_2^- is 10 - 20 nM (Misko et al. 1993). However, there is difficulty in employing the batch fluorometric method to detect picomolar levels of NO_2^- and NO_3^- in cell culture medium and biological samples because of high background absorbance as well as the fluorescence quenching and interference by biological components and colorimetric chemicals (Li et al. 2000).



2,3-diaminonaphthalene 2,3-naphthotriazole

Figure 7.1. Reaction of nitrite with 2,3-diamoninaphthalene (DAN) to form 2,3-naphthotriazole (NAT).

Our literature survey showed that most of the reports published on the measuring of NO in cell culture medium were carried out in isolated macrophages or macrophage cell lines that produce NO in the micromolar range (Akaike et al. 1997; Li et al. 2000; Meyer et al. 1997; Muscara and de Nucci 1996). The objective of this study was to develop a feasible and more sensitive method for measuring NO in murine hepatoma Hepa 1c1c7 cells. In this study, we showed for the first time that we could measure NO in Hepa 1c1c7 cells at picomolar levels. Thus, this method offers high sensitivity for determining picomolar levels of NO_2^- in cell culture medium. In addition, we compared our HPLC method with most commonly used method, the Griess method, to measure NO_2^- in Hepa 1c1c7 cells.

Materials and Methods

Materials

HPLC-grade acetonitrile, methanol and water were purchased from Fisher Scientific (Edmonton, AB, Canada). DAN was obtained from ICN Biomedicals (Costamesa, California, USA). Cell culture materials: L-glutamine, gentamycin sulfate,

penicillin, streptomycin, amphotericin B, and fetal bovine serum were obtained from Invitrogen (Burlington, ON, Canada). Recombinant murine tumor necrosis factor-α (TNF-α) was purchased from Peprotech Canada (Ottawa, ON, Canada). All other chemicals, which were analytical grade, were obtained from Sigma-Aldrich (St. Louis, MI, USA).

Cell culture and treatments

Hepa 1c1c7 cell lines (generously provided by Dr. O. Hankinson, University of California, Los Angeles, USA) were maintained in standard media consisting of Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum, 20 μM L-glutamine, 50 μg/ml gentamycin sulfate, 100 IU/ml penicillin, 10 μg/ml streptomycin, and 25 ng/ml amphotericin B. Cells were grown in 75-cm^2 rectangular canted neck cell culture flask with vent cap at 37°C in a 4% CO_2 humidified environment. The culture media was changed every 2 days in order to keep the pH at 7.4 . Before culturing into 6-well sterile cell culture plates they were washed with phosphate buffered saline, trypsinized and re-suspended in Dulbecco's modified eagle medium containing 10% fetal bovine serum. 2 ml of this stock was transferred into each well of the 6-well cell culture plate. After 24 h incubation in a humidified incubator in 5% CO_2 at 37°C, the medium in the 6-well cell culture plate was discarded, each well washed with 0.5 ml phosphate buffered-saline and 0.5 ml of Hanks' balanced salt solution (1.26 mM $CaCl_2.2H_2O$, 5mM KCl, 0.4 mM KH_2PO_4, 0.8 mM $MgSO_4.7H_2O$, 137 mM NaCl, 4 mM $NaHCO_3$, 0.3 mM $Na_2HPO_4.7H_2O$, 5.5 mM glucose) was added into each wells of the 6-well cell culture plate. Then the plates were incubated with vehicle or TNF-α (10 ng/ml) in the humidified

incubator in 5% CO_2 at 37°C for 10 hours. Thereafter, Hanks' balanced salt solution or cells were collected and stored at -20°C until analyzed for their NO_2^- and protein content, respectively. Cellular protein content was measured using the Lowry method (Lowry et al. 1951).

Griess assay

The amount of NO_2^- in the Hanks' balanced salt solution because of the stimulation of the Hepa 1c1c7 cells with TNF-α was determined by Griess method (Schmidt 1995). Briefly, using 96-well cell culture plates, 100 µl of vehicle or sample was mixed with 100 µl of the Griess reagent (0.1 g of sulphanilamide dissolved in 10 ml of prewarmed 2.5% H_3PO_4 mixed with 0.03 g of naphthylethylenediamine) in 10 ml of 2.5% H_3PO_4. After 10 minutes at room temperature, the optical density of the samples was measured using Bio-Tek El-312e microplate reader (Winooski, VT, USA) at 560 nm.

HPLC System

The system consisted of a Sil-9A model autosampler (Shimadzu, Japan), a Waters 470 model fluorescence detector set at 375nm as excitation and 415nm as emission (Waters, Mississauga, ON, Canada) and a 3390A model integrator (Hewlett Packard, USA). The mobile phase, 60% of 15 mM sodium phosphate buffer (pH 7.5) and 40% methanol, was pumped through the system using a Waters 501 model HPLC pump (Waters, Mississauga, ON, Canada) at a flow rate of 1 ml/min. A 10-µm reversed-phase C18 column (250 x 4.00 mm, I.D.) (Phenomenex, Torrance, CA, USA) and HPLC guard-column insert packed with C18 (Waters, Mississauga, ON, Canada) were used for the

assay. 30 µl of the derivatized nitrite - DAN solution was injected into the HPLC system at room temperature.

HPLC analysis of nitrite

For preparing the samples, 100 µl of NO_2^- standard dissolved in Hanks' balanced salt solution or cells treated in Hanks' balanced salt solution were diluted 1:3 with acetonitrile, vortex-mixed for 30 seconds and then centrifuged at 12,000 g for 3 min to precipitate the protein. Thereafter, the supernatant was aspirated and the extra solvent was evaporated (Savant Speed Vac concentrator-evaporator, Emerson Instruments Scarborough, ON, Canada). The residue was reconstituted in 100 µl of double distilled water and used for HPLC analysis. Hanks' balanced salt solution was used because we found that DAN reacts with the Dulbecco's modified eagle medium contents and results in peaks that interfere with our NAT.

At room temperature, DAN reacts rapidly with NO_2^- under acidic conditions to form the highly fluorescent product NAT (Hampl et al. 1996) (Figure 7.1), which is stable in alkaline solution (Sawicki 1971). Fluorescence is monitored following the addition of NaOH, which raises pH and increases the sensitivity (Hampl et al. 1996). For HPLC analysis of NO_2^-, 100 µl of NO_2^- standard (0-200 pM) or samples, all reconstituted in double-distilled water were incubated in the dark at 25°C with 10 µl of freshly prepared DAN (1 µg/ml in 0.62 M HCl) for 10 min, followed by addition of 5 µl of 2.8M NaOH. This reaction mixture was directly used for the chromatographic analysis.

Accuracy and Precision

Different concentrations of standard NO_2^- (10, 25, 50, 100 and 200 pM) along with blank of Hanks' balanced salt solution were prepared in triplicate on three consecutive days. The accuracy (the nearness of a measured value to the true value) was expressed as the mean percentage error, [(mean measured concentration)/(expected concentration)] x 100. The precision (agreement between replicate measurements) was evaluated as inter and intra-day coefficient of variation by the relative deviation [%CV = (SD/mean) x 100]. The least-squared regression method was used to determine the regression coefficient and the equation for the best fitting line.

Statistical analysis

The comparison of the results from the experimental groups and its control was carried out by One-way analysis of Variance (ANOVA) followed by the Newman-Keuls *post hoc* test.

Results

Typical chromatographs of NO_2^-, obtained after direct injection of standard, untreated cells or cells treated with TNF-α are presented in Figure 7.2. NAT appeared on the chromatograph in approximately 16 min with no interference peaks. Under our experimental conditions, DAN did not interfere with NAT (Figure 7.2). The fluorescence intensity for NAT, at excitation at 375 nm and emission at 415 nm, is at least 90-100 fold higher than that observed for an equimolar concentration of DAN (Misko et al. 1993). It is important to mention that fluorescent substances present in the cell culture medium

(Dulbecco's modified eagle medium) were eliminated in our method by using the Hanks' balanced salt solution. Thus, our HPLC method offers greater specificity for determining NO_2^- in cell culture than all other fluorometric methods used previously.

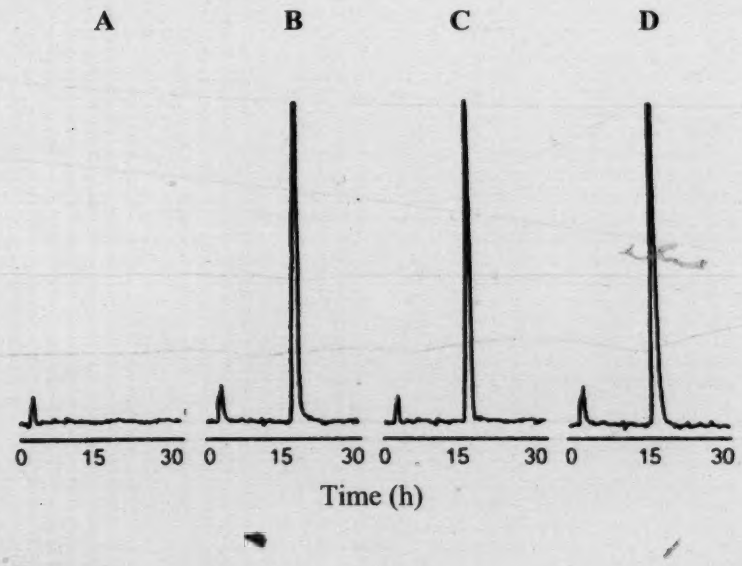


Figure 7.2. Representative 30 min chromatographs of NAT: A) Hanks' balanced salt solution with DAN, B) Standard, 200 pM nitrite, C) Untreated cells, 137 pM nitrite, D) TNF-α-treated cells, 279 pM nitrite.

In this HPLC method, excellent linearity was observed between the peak area and NO_2^- concentration over the range of 10 to 200 pM (r^2 >0.99). The detection limit for NO_2^- was 8 pM, based on a signal-to-noise ratio of 3, when using the Waters 470 Fluorescence Detector at a gain setting of 1000. Inter and Intra-day reproducibility were determined using different concentrations of standard NO_2^- (10, 25, 50, 100 and 200 pM). These concentrations were assayed in triplicate on three consecutive days. Inter- and intra-day reproducibility characterized by CV did not exceed 11% and 10%

respectively. In addition, the accuracy of the assay was above 90% (Table 1). Incubation of Hepa 1c1c7 cells for 10 hours in the presence of TNF-α (10 ng/ml), which lead to a marked expression of iNOS and NO formation (Morris and Billiar 1994; Pittner and Spitzer 1992), resulted in the formation of NO_2^- in picomolar range as determined by our fluorescence HPLC analysis (Figure 7.3), thereby improving the specificity and sensitivity of the HPLC method for analysis of NO_2^- in cell culture medium. These data indicate the high reproducibility and reliability of the method for determining NO_2^-. NO production by TNF-α-treated cells is estimated to be approximately two fold higher than that of untreated cells ($p<0.05$) (Figure 7.3). Using the Griess colorimetric assay, we failed to detect NO_2^- in Hepa1c1c7 wild-type cells.

Table 6.1: Assay validation data, inter-day and intra-day precision and accuracy

Inter-day precision (coefficient of variation, CV) and accuracy for NO assay with HPLC (n=3)
Concentration (pM)

Actual	Observed	CV (%)	Accuracy (%)
10	9.2	5.4	92 ± 5.4
25	26.0	3.2	104 ± 3.2
50	46.4	10.9	92 ± 10.9
100	105.0	2.8	105 ± 2.8
200	198.2	0.6	99 ± 0.6

Intra-day precision (coefficient of variation, CV) and accuracy for NO assay with HPLC (n=3)
Concentration (pM)

Actual	Observed	CV (%)	Accuracy (%)
10	9.8	8.7	98 ± 8.7
25	26.2	9.4	105 ± 9.4
50	45.5	6.2	91 ± 6.2
100	107.5	2.3	107 ± 2.3
200	197.6	5.6	99 ± 5.6

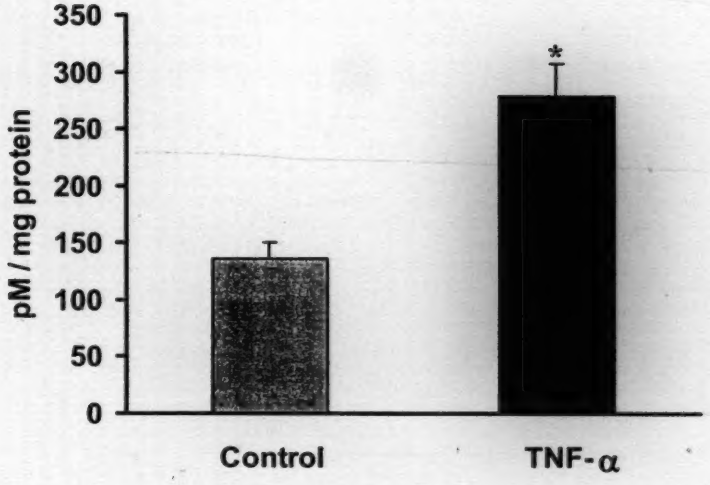


Figure 7.3. Determination of nitrite in Hepa 1c1c7 cells with the fluorometric HPLC method. Cells were treated with TNF-α (10 ng/ml) in Hanks' balanced salt solution for 10 hrs prior to assay. Data are expressed as mean ± SD (n = 6), *p<0.05 compared to control.

Discussion

The formation of NO can result in several reactive nitrogen intermediates that can be determined by various detection methods (Stratford et al. 1997). Although many recent publications substantiate this interest, there is no work to our knowledge that measures picomolar levels of NO_2^- in Hepa 1c1c7, one of the most common cell types used in drug metabolism studies. Therefore, the goal of the present study was to develop a simple method to measure NO_2^- in this cell line.

The low detection limit of the Griess reagent and other common methods for measuring NO_2^- in cell culture systems has hampered attempts to study the precise metabolic pathways of NO during cellular damage. Several attempts have recently been made to measure NO_2^- levels in the nanomolar range using expensive or difficult techniques. However, while these techniques require expensive equipment, HPLC systems are available in most laboratories. HPLC is a convenient and rapid technique for the separation of a wide variety of chemical species, including biological molecules, and can be employed for the determination of NO_2^-.

In the present study, we demonstrated that the activation of Hepa 1c1c7 cells with TNF-α results in a marked increase in NO formation. In contrast to the Griess method, this HPLC method can be used to measure NO_2^- production in the picomolar range. The sensitivity of this HPLC method for NO_2^- analysis is greater than that of the batch fluorometric method (detection limit, 10-20 μM), the Griess colorimetric assay (detection limit 0.1 - 1 μM) (Misko et al. 1993; Nagano 1999), and the HPLC methods with UV-VIS or conductimetric detection (detection limit, 0.1-0.5 μM) (Marzinzig et al. 1997;

Wiersma 1970). Moreover, we found that the samples prepared by our method could be used directly for NO_2^- analysis by the HPLC method without prior filtration. With this method, we are also able to eliminate the high background absorbance caused by the contents of cell culture medium, Dulbecco's modified eagle medium.

In conclusion, the fluorometric HPLC method described here offers high sensitivity, reproducibility and specificity for measuring picomolar levels of NO_2^-. Moreover, the simple sample preparation with this method makes it particularly suitable for routine analysis of a large series of samples. The measurement of NO synthase activity in biological systems has attained increasing importance as the enzyme's involvement in both normal and pathological process is elucidated (Moncada et al. 1991; Nathan 1992; Southan and Szabo 1996; Xie et al. 1992).

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8. ROLE of NITRIC OXIDE in DOWN-REGULATION of CYTOCHROME P450 1A1 and NADPH: QUINONE OXIDOREDUCTASE 1 by TUMOR NECROSIS FACTOR-α and LIPOPOLYSACCHARIDE

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Introduction

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that controls the expression of different genes whose functions are associated with oxidative stress response or linked to the metabolism of dietary constituents, drugs and environmental contaminants (Burbach et al. 1992; Elbekai et al. 2004; Ema et al. 1992; Gharavi and El-Kadi 2005a). Upon ligand binding, the AhR-ligand complex translocates from cytoplasm to the nucleus. Then, the activated AhR dimerizes with the AhR nuclear translocator protein (ARNT), and binds to a class of promoter DNA sequences called xenobiotic responsive element (XRE) of the target genes to activate their transcription (Nebert et al. 1993; Whitlock 1999). The AhR-regulated genes consist of four phase I enzymes [cytochrome P450 1A1 (CYP1A1), CYP1A2, CYP1B1, and CYP2S1] and four phase II xenobiotic metabolizing enzymes, including NADP(H):quinone oxidoreductase 1 (NQO1), glutathione S transferase A1 subunit (GSTA1), cytosolic aldehyde dehydrogenase-3 (ALDH3), and UDP glucuronosyltransferase 1A6 (UGT1A6) (Nebert and Duffy 1997; Rivera et al. 2002).

In humans and animals, cytochrome P450 (CYP) and their activities are usually suppressed by infections and other inflammatory stimuli, though some are unaffected or may be induced (Morgan 1997). CYPs suppression can result in increased clinical toxicity of drugs with low therapeutic indices (Morgan 1997). Conversely, suppression of the metabolism during an inflammation, may lead to reduced therapeutic or toxic effects of drugs that are metabolized to pharmacologically or toxicologically active compounds. These changes in CYPs have been linked to increase serum concentrations of

proinflammatory cytokines including tumor necrosis factor-α (TNF-α) interleukin-1 (IL-1), and IL-6 (Abdel-Razzak et al. 1993; Bleau et al. 2001; Bleau et al. 2000; El-Kadi et al. 2000; El-Kadi and du Souich 1998; Fukuda et al. 1992). We have previously shown that TNF-α and lipopolysaccharide (LPS) down-regulated the constitutive expression and the AhR ligand-mediated induction of Cyp1a1 and Nqo1 activities as well as expression, while the underlying mechanisms remain unknown (Gharavi and El-Kadi 2005b).

The role of nitric oxide (NO) in the regulation of CYP expression during inflammation has been the subject of debate. Various studies using whole animals (Khatsenko and Kikkawa 1997; Khatsenko et al. 1998), microsomes (Minamiyama et al. 1997; Wink et al. 1993) or hepatocytes (Carlson and Billings 1996; Stadler et al. 1994) have suggested that the production of NO during inflammatory responses leads to the down-regulation of CYP. Some studies have also suggested that NO-derived product, peroxynitrite ($ONOO^-$) which can be formed by a rapid reaction of NO and superoxide may be responsible for this down-regulation (Roberts et al. 1998). Although these studies implicated the role of NO in CYP down-regulation several recent studies reported that NO is not required for the suppression of CYP by LPS or cytokines (Sewer et al. 1998; Sewer and Morgan 1997; Sewer and Morgan 1998). In support for the NO-independence suppression of CYP several studies have shown that inducible nitric oxide synthase (NOS2) inhibition had no effect on cytokine-mediated down-regulation of CYP-catalyzed activities (Hodgson and Renton 1995; Monshouwer et al. 1996). On the other hand, with respect to phase II xenobiotic metabolizing enzymes, it has been reported that Nqo1 is sensitive to nitrosative stress and Nqo1 expression can be induced by NO through

antioxidant response element (ARE) (Dhakshinamoorthy and Porter 2004). However, little is known about the role of NO in the down-regulation of Nqo1-mediated by inflammation. Therefore, in the present study we investigated the role of NO and peroxynitrite in the suppression of *Cyp1a1* and *Nqo1* gene expression by inflammation in murine hepatoma Hepa 1c1c7 cells.

Materials and methods

Tumor necrosis factor-α was obtained from Peprotech Canada (Ottawa, ON). Lipopolysaccharide, β-naphthoflavone, nicotinamide adenine dinucleotide phosphate, 2,6-dichlorophenolindophenol, flavin adenine dinucleotide, 7-ethoxyresorufin, bovine serum albumin, Dulbecco's modified Eagle's medium base and glucose were purchased from Sigma-Aldrich (St. Louis, MI). Resorufin, 100X vitamin supplement solution and amphotericin B were purchased from ICN Biochemicals Canada (Toronto, ON). L-N6-(1-iminoethyl) lysine was obtained from Cedarlane Lab (Homby, ON). Iron tetrakis (N-methyl-4'-pyridyl) porphyrinato was from EMD Biosciences, Inc. (San Diego, CA). Penicillin/streptomycin, gentamicin, L-glutamine, fetal bovine serum, non-essential amino acid solution, TRIzol reagent and the random primers DNA labeling system were obtained from Invitrogen Canada (Burlington, ON). [α-^{32}P]dCTP (3000 Ci/mmol) was supplied by Perkin-Elmer (Boston, MA). All other chemicals were purchased from Fisher Scientific (Toronto, ON).

Cell culture and treatments

Murine hepatoma Hepa 1c1c7 cells (generously provided by Dr. O. Hankinson, University of California, Los Angeles) were maintained in Dulbecco's Modified Eagle's Medium, without phenol red, supplemented with 10% fetal bovine serum, 20 μM L-glutamine, 50 μg/ml gentamicin sulfate, 100 IU/ml penicillin, 10 μg/ml streptomycin and 25 ng/ml amphotericin B. Cells were grown in 75-cm² rectangular canted neck cell culture flask with vent cap at 37°C in a 4% CO_2 humidified environment. The culture media was changed every 2 days in order to keep the pH at 7.4.

TNF-α was initially reconstituted with 0.1% bovine serum albumin and made up to the desired final concentration with sterile phosphate buffered-saline, pH 7.4. LPS was dissolved in sterile PBS and β-naphthoflavone (βNF) was dissolved in dimethylsulfoxide. L-N6-(1-iminoethyl) lysine (L-NIL), iron tetrakis (N-methyl-4'-pyridyl) porphyrinato (FeTMPyP) were dissolved in sterile distilled water. For all experiments, control group for each of the treatment was administered the appropriate solvent. For analysis of mRNA and Nqo1 activity, ~1 × 10^6 cells were added to a 6-well tissue culture plate in 2 ml of culture media. For analysis of Cyp1a1 activity, ~1 × 10^6 cells were added to each well of a 96-well tissue culture plate in 200 μl of culture media. On 60-80% confluence (1-2 days), appropriate stock solutions of the test chemicals were directly added to the culture media. L-NIL and FeTMPyP were added 2 h before treatment of cells with TNF-α or LPS.

Measurement of NO production

The amount of NO produced by various concentrations of TNF-α or LPS in Hepa 1c1c7 was measured by the validated fluorometric high performance liquid chromatographic (HPLC) assay as described previously (Gharavi and El-Kadi 2003). Briefly, cells were incubated with vehicle, TNF-α (1, 5 and 10 ng/ml) or LPS (1 and 5 μg/ml) in Hanks' balanced salt solution for 10 h. NO production was determined by measuring the conversion of 2,3-diaminonaphthalene to its fluorescent product, 2, 3-naphthotriazole. 2, 3-naphthotriazole was analyzed after elution with 60% of 15 mM sodium phosphate buffer (pH 7.5) and 40% methanol through a 10-μm reversed-phase C18 column (250 x 4.00 mm, I.D.) at a flow rate of 1 ml/min. Fluorescence was monitored with excitation at 375 nm and emission at 415 nm.

Determination of Cyp1a1 activity

Cyp1a1-dependent 7-ethoxyreorufin *O*-deethylase (EROD) activity was measured in on intact, living cells as described previously (Gharavi and El-Kadi 2005b). Enzymatic activity was normalized for cellular protein content which was determined using a modified fluorescent assay (Gharavi and El-Kadi 2005b).

Preparation of cell lysates for enzyme activity assay

Twenty four hours after incubating the cells with the tested compounds, cultured cells were washed with phosphate buffer saline then 0.5 ml of homogenization buffer (50 mM potassium phosphate, 1.15% KCL, pH 7.4) was added to each well of a 6-well plate. The plates were frozen for 24 h in -80°C freezer. Thawed cells were homogenized with a

Kontes homogenizer and then centrifuged at 5000 x *g* for 20 min. The supernatant fractions were then removed and protein concentrations in the supernatant were determined by the method of Lowry (Lowry et al. 1951), using bovine serum albumin as the standard. The prepared samples were stored in -20°C freezer until they were used in the determination for Nqo1 enzyme activity.

Determination of Nqo1 activity

Nqo1 activity was determined by the continuous spectrophotometric assay of Ernster (Ernster 1967), which quantitates the dicoumarol-inhibitable reduction of 2,6-dichlorophenolindophenol. The rate of reduction of 2,6-dichlorophenolindophenol (40 μM) by NADPH (200 μM) in 1 ml of Tris-HCl buffer (pH 7.8, 25 mM) containing 0.1% Tween-20 and 0.023% BSA was monitored for 2 min at 600 nm with $\varepsilon = 2.1\ mM^{-1}\ cm^{-1}$.

RNA extraction and Northern blot analysis

After incubation of the cells with the test compounds for the specified time periods, total cellular RNA was isolated using TRIzol reagent, according to manufacturer's instructions (Invitrogen), and quantified by measuring the absorbance at 260 nm. Northern blot analysis of total RNA was performed as described elsewhere (Gharavi and El-Kadi 2005b).

The mouse Cyp1a1 and Gapdh cDNA probes were generously provided by Dr. John R. Bend (University of Western Ontario, London, ON). The cDNA probe for mouse Nqo1 mRNA was prepared as previously described (Chen et al. 1994). All probes were

^{32}P-labeled by the random primer method according to the manufacturer's (Invitrogen) instructions. The intensities of the Cyp1a1 and Nqo1 mRNAs were quantified, relative to the signals obtained for Gapdh mRNA, using a Java-based image processing software, ImageJ [W. Rasband (2005) National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij.].

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of mRNA

RT-PCR was performed using murine leukemia virus reverse transcriptase (Invitrogen) as described previously with slight modifications (Chen et al. 2004). Briefly, 1 μg of purified total RNA was used in 40 μL RT reaction containing 8 μM oligo (dT), 1 mM dNTP, 40 U RNase, 10 mM dithiothreitol, and 400 U reverse transcriptase (Invitrogen). The reaction was incubated at 37° for 10 min and 42° for 1 h, then heated to 70° for 15 min to inactivate the RT. 2.5 μl of RT reaction mixture was subjected to PCR amplification with 1 mM specific forward and reverse primers for inducible nitric oxide (NOS2) (0.25 μM for HPRT) in a 50 μL reaction containing 0.2 mM dNTPs, 2 U Taq polymerase (Invitrogen), and 1.5 mM $MgCl_2$ for NOS2 (2.5 mM for HPRT). PCR was performed using AmpliTAQ DNA Polymerase (Perkin–Elmer) according to the manufacturer's instructions. cDNA for NOS2 was denatured at 95° for 5 min and cycled immediately 30 times at different temperatures including, 95° for 20 sec (denaturation), 55° for 25 sec (annealing), and 72° for 45 sec (extension). The PCR reaction ended with 10 min elongation step at 72°. HPRT was used as the internal standard to normalize for RNA loading and PCR variation and its amplifications were run for 5 min at 94° followed by 30 cycles consisting of 1 min for denaturation at 94°, 1 min 30 sec for

annealing, and 1 min for extension at 72°. The PCR reaction ended with 10 min elongation step at 72. 1.5% agarose gel electrophoresis was used to separate PCR-amplified products. Ethidium bromide was added to the gel before solidification. PCR products were visualized under a UV transluminator and digitally recorded. Band intensity was quantitated using a Java-based image processing software, ImageJ [W. Rasband (2005) National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij.]. Primer sequences used were: NOS2 forward, 5'-CTGCAGCACTTGGATCAGGAACCTG-3'; reverse, 5'-GGGAGTAGCCTGTGTGCACCTGGAA-3' and HPRT forward, 5'-GCTGGTGAAAAGGACCTCT-3'; reverse, 5'-CACAGGACTAGAACACCTGC-3' as described previously (Chen et al. 2004; Ke et al. 2001).

Statistical Analysis

All results are presented as mean ± SD (standard deviation). The comparison of the results from the various experimental groups and their corresponding controls was carried out by a one way analysis of variance (ANOVA) followed by Student-Newman-Keuls *post hoc* tests. The differences were considered significant when $p<0.05$.

Results

Time-dependent effect of TNF-α and LPS on the inducible expression of Cyp1a1 and Nqo1 mRNA

To examine the effect of TNF-α and LPS on the kinetics of Cyp1a1 and Nqo1 mRNA, Cyp1a1 and Nqo1 mRNA contents were measured at various time points (0, 1, 3,

6, 12, and 24 h) following the incubation of Hepa 1c1c7 cells with vehicle, TNF-α (10 ng/ml) and LPS (5 μg/ml) in the presence of βNF (10 μM). As shown in Figure 8.1, the βNF-inducible Cyp1a1 mRNA was detectable as early as 1 h and remained elevated for at least 24 h. The maximal induction was occurred at 3 h, followed by 87% drop of the maximal level at 24 h. A densitometric scan of the autoradiogram indicates that TNF-α and LPS significantly decreased the steady-state of βNF-inducible Cyp1a1 mRNA level by 56 and 42%, respectively. On the other hand, Figure 8.2 shows that the onset of Nqo1 mRNA induction mediated by βNF occurred at 3 h and reached the maximal level at 6 h, followed by a 90% decline of its maximal level at 24 h. When the cells were co-treated with βNF and either TNF-α or LPS, the steady-state mRNA levels of Nqo1 was decreased by approximately 61 and 39%, respectively. Taken together, the decrease in the steady-state of Cyp1a1 and Nqo1 mRNA levels by TNF-α and LPS reflects the down-regulation of these genes.

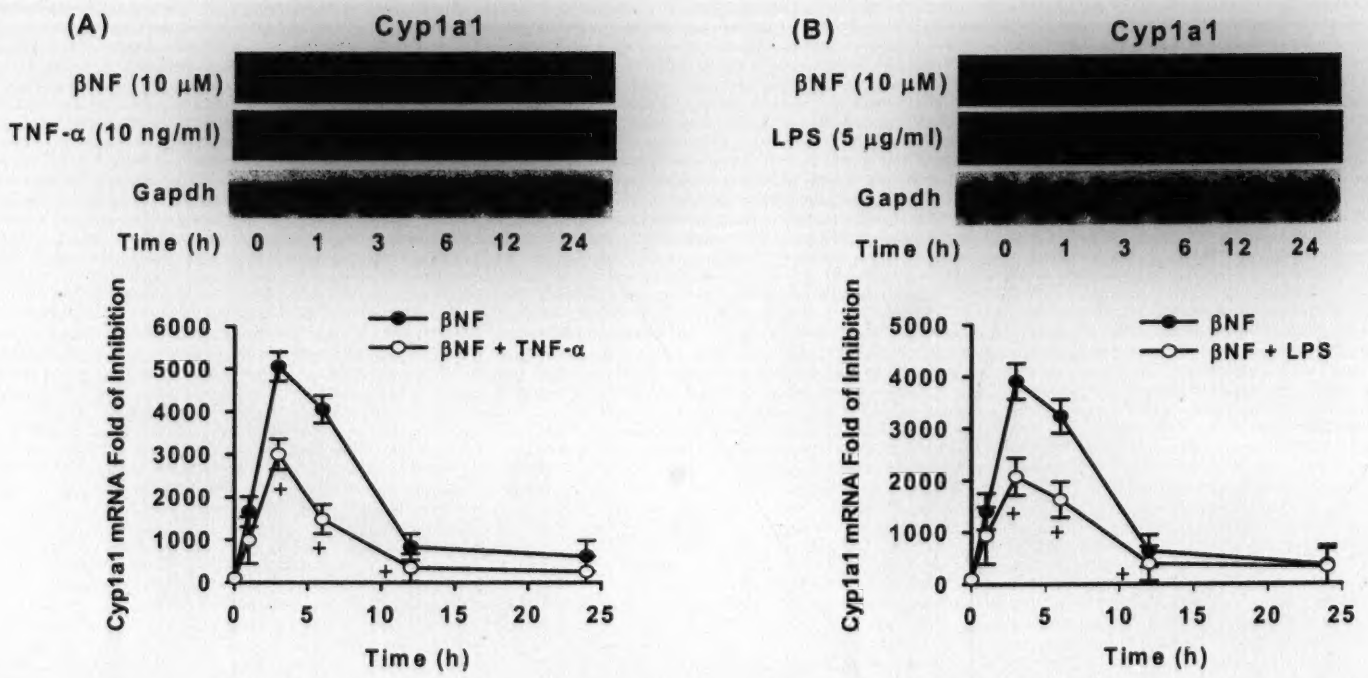

Figure 8.1. Time-dependent effects of TNF-α (A) and LPS (B) on the inducible expression of Cyp1a1 mRNA. Cells were treated with βNF (10 μM) in the absence or presence of TNF-α (10 ng/ml) or LPS (5 μg/ml) at different time (0, 1, 3, 6, 12, and 24 h) prior to Northern blot analysis. The graph represents the relative normalized amount of Cyp1a1 mRNA (mean ± SD, n =3) expressed as relative Cyp1a1 mRNA level, which was quantified by ImageJ software and normalized to Gapdh levels. + p<0.05 compared to βNF.

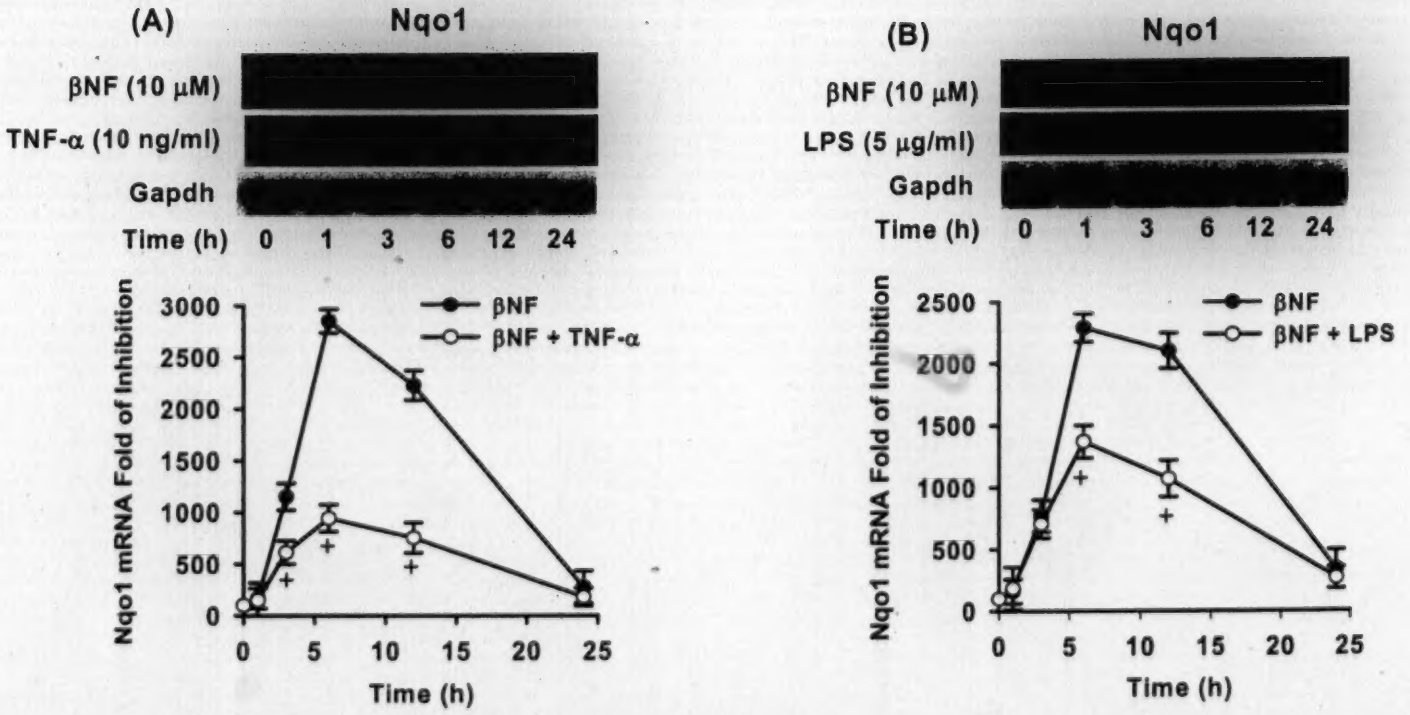

Figure 8.2. Time-dependent effects of TNF-α (A) and LPS (B) on the inducible expression of Nqo1 mRNA. Cells were treated with βNF (10 μM) in the absence or presence of TNF-α (10 ng/ml) or LPS (5 μg/ml) at different time (0, 1, 3, 6, 12, and 24 h) prior to Northern blot analysis. The graph represents the relative normalized amount of Nqo1 mRNA (mean ± SD, n =3) expressed as relative Nqo1 mRNA level, which was quantified by ImageJ software and normalized to Gapdh levels. + p<0.05 compared to βNF.

Effect of NOS2 inhibitor, L-NIL, on TNF-α and LPS-mediated NO production

As shown in Figure 8.3, treatment of Hepa 1c1c7 cells with TNF-α (1, 5 and 10 ng/ml) or LPS (1 and 5 μg/ml) resulted in a significant increase of NO production compared to control. Various concentrations of TNF-α, but not LPS, caused a concentration-dependent production of NO. Pre-treatment of the cells with the NOS2 inhibitor, L-NIL (1 mM) (Banan et al. 2001; Fiorucci et al. 2001), for 2 h significantly inhibited the TNF-α-mediated NO production only at 10 ng/ml, whereas LPS-mediated NO production was inhibited at both concentrations tested (1 and 5 μg/ml).

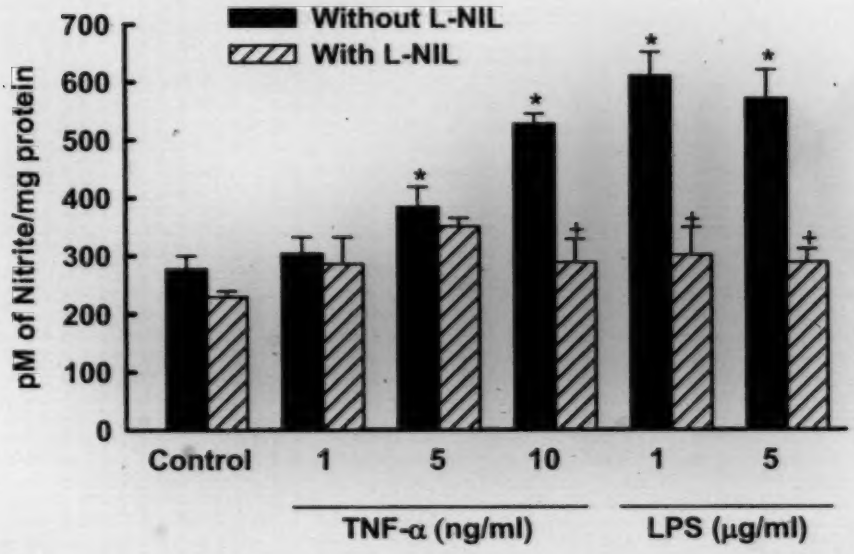

Figure 8.3. Effect of L-NIL on the TNF-α or LPS-mediated NO production. Cells were treated with different concentrations of TNF-α (1, 5 and 10 ng/ml) or LPS (1 and 5 μg/ml) in the absence or presence of L-NIL (1 mM) for 12 h prior to HPLC assay. Data are expressed as mean ± SD (n = 6), *p<0.05 compared to control, +p<0.05 compared to the cells without L-NIL.

Effect of TNF-α and LPS on the expression of NOS2 mRNA

RT-PCR assay was performed to assess whether the increase in NO production by TNF-α or LPS was accompanied by an increase in the expression of NOS2 mRNA. For this purpose, Hepa 1c1c7 cells were incubated with various concentrations of TNF-α (1, 5 and 10 ng/ml) or LPS (1 and 5 μg/ml) for 6 h. Our results demonstrate that TNF-α significantly induced the NOS2 mRNA expression in a concentration-dependent manner. Also, LPS induced the NOS2 mRNA expression, although the increase did not appear to be concentration-dependent. These data paralleled the effect of TNF-α and LPS on NO production (Figure 8.4)

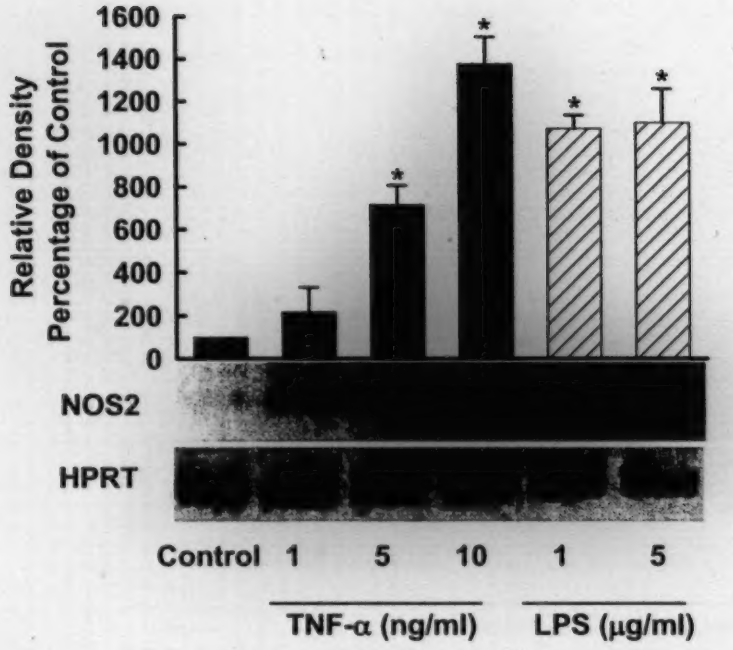

Figure 8.4. Effect of TNF-α and LPS on the expression of NOS2 mRNA. Cells were treated with TNF-α (1, 5 and 10 ng/ml) or LPS (1 and 5 μg/ml) for 6 hr prior to RT-PCR analysis. Data are expressed as mean ± SD ($n = 3$), *$p<0.05$ compared to control.

Effect of NOS2 inhibitor, L-NIL, on TNF-α and LPS-mediated down-regulation of inducible expression of Cyp1a1 and Nqo1

To determine whether NO is responsible for the down-regulation of Cyp1a1 and Nqo1 by TNF-α or LPS, Hepa 1c1c7 cells were pre-incubated with the NOS2 inhibitor, L-NIL (1 mM) for 2 h prior to the addition of TNF-α (1, 5 and 10 ng/ml) or LPS (1 and 5 μg/ml) in the presence of βNF (10 μM). As shown in Figure 8.5, pre-treatment of Hepa 1c1c7 cells with L-NIL partially prevented the TNF-α and LPS-mediated down-regulation of Cyp1a1 mRNA at the highest concentration tested for TNF-α (10 ng/ml) and LPS (5 μg/ml) by 10 and 17%, respectively. At the activity level, L-NIL also partially prevented the decrease in βNF-mediated the induction of Cyp1a1 activity at 5 and 10 ng/ml for TNF-α and 5 μg/ml for LPS (Figure 8.6). With respect to Nqo1, pre-treatment of Hepa 1c1c7 cells with L-NIL did not significantly alter the down-regulation of Nqo1 expression mediated by TNF-α and LPS at both mRNA and activity levels (Figure 8.7 and 8.8). Our result indicates that NO is involved in the TNF-α and LPS-mediated the down-regulation of Cyp1a1 but not Nqo1.

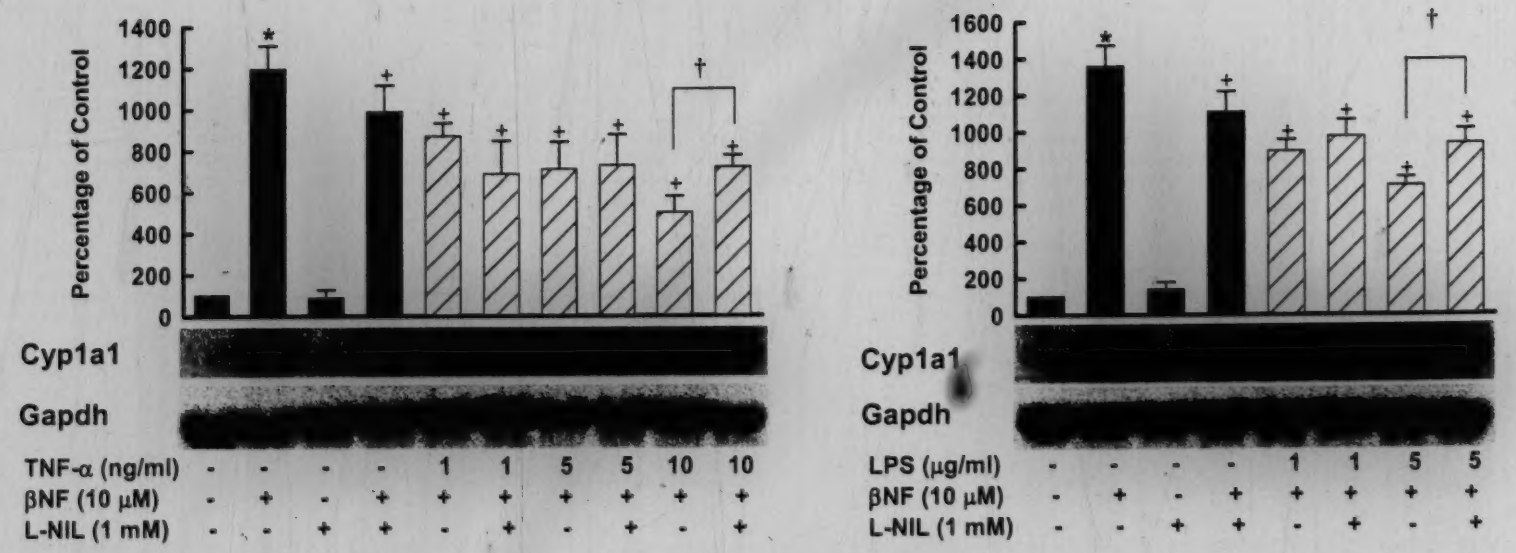

Figure 8.5. Effect of TNF-α and LPS on the βNF-mediated induction of Cyp1a1 mRNA in the absence or presence of L-NIL. Cells were treated with βNF (10 μM) and TNF-α (1, 5 and 10 ng/ml) or βNF (10 μM) and LPS (1 and 5 μg/ml) in the absence or presence of L-NIL (1 mM) for 6 h prior to Northern blot analysis. The graphs represent the relative normalized amount of Cyp1a1 mRNA (mean ± SD, n = 3), expressed as percentage of the control, which was quantified by ImageJ software and normalized to Gapdh levels. *p<0.05 compared to control, +p<0.05 compared to the cells treated with βNF.

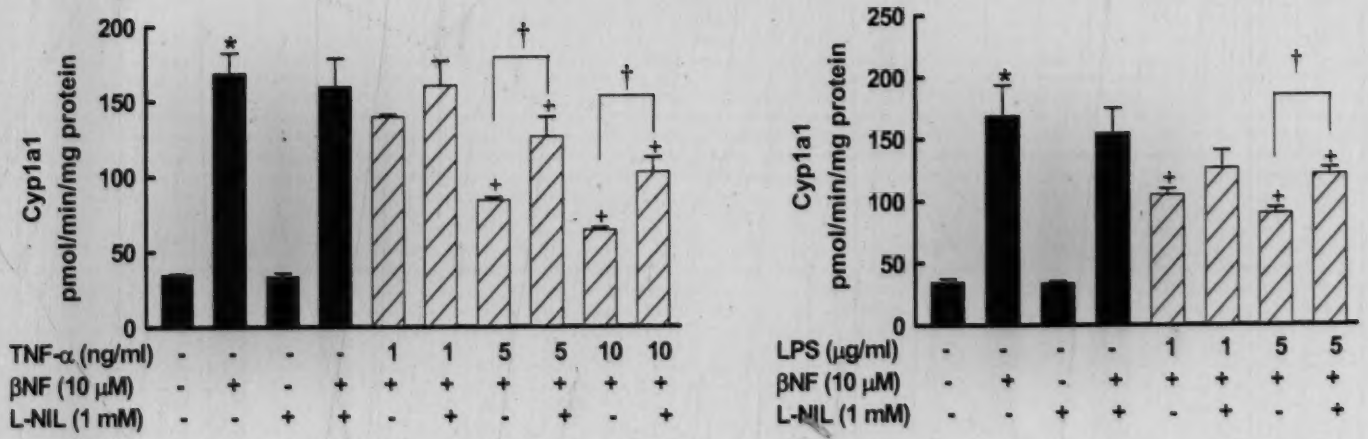
Cyp1a1
pmol/min/mg protein
200
150
100
50
0
TNF-α (ng/ml) - - - - 1 1 5 5 10 10
βNF (10 μM) - + - + + + + + + +
L-NIL (1 mM) - - + + - + - + - +
Cyp1a1
pmol/min/mg protein
250
200
150
100
50
0
LPS (μg/ml) - - - - 1 1 5 5
βNF (10 μM) - + - + + + + +
L-NIL (1 mM) - - + + - + - +

Figure 8.6. Effect of TNF-α and LPS on the βNF-mediated induction of Cyp1a1 activity in the absence or presence of L-NIL. Cells were treated with βNF (10 μM) and TNF-α (1, 5 and 10 ng/ml) or βNF (10 μM) and LPS (1 and 5 μg/ml) in the absence or presence of L-NIL (1 mM) for 24 h prior to enzyme activity assay ($n = 6$), respectively. Data are expressed as mean ± SD, *$p<0.05$ compared to control, +$p<0.05$ compared to the cells treated with βNF.

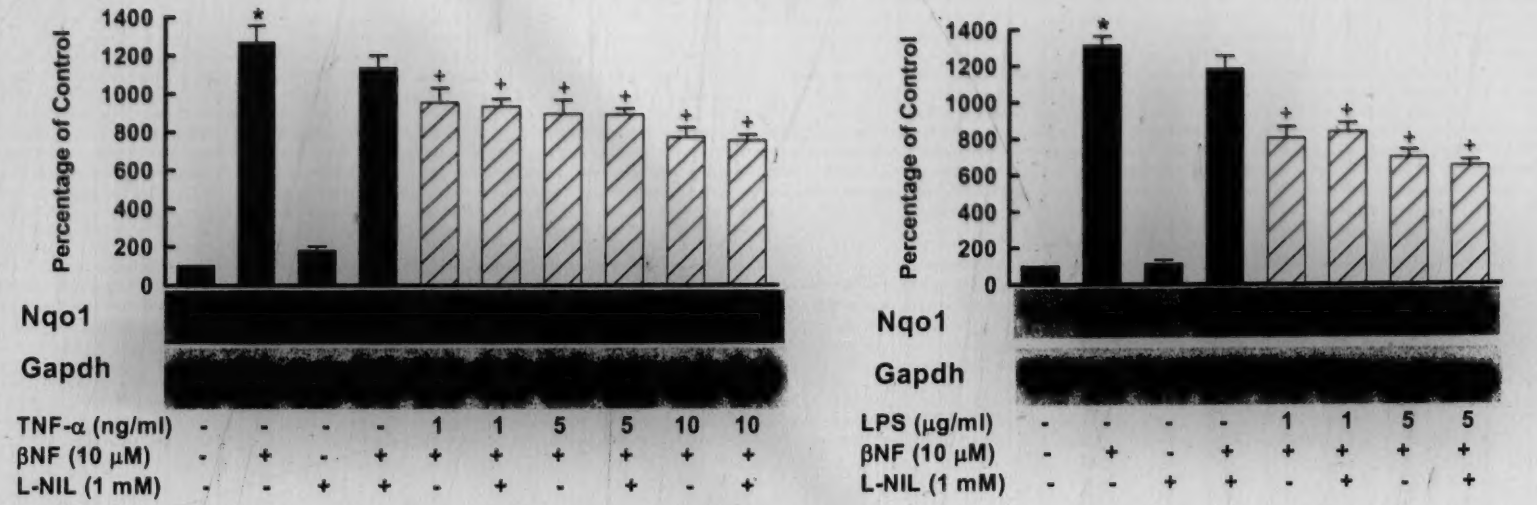

Figure 8.7. Effect of TNF-α and LPS on the βNF-mediated induction of Nqo1 mRNA in the absence or presence of L-NIL. Cells were treated with βNF (10 μM) and TNF-α (1, 5 and 10 ng/ml) or βNF (10 μM) and LPS (1 and 5 μg/ml) in the absence or presence of L-NIL (1 mM) for 6 h prior to Northern blot analysis. The graphs represent the relative normalized amount of Nqo1 mRNA (mean ± SD, n = 3), expressed as percentage of the control, which was quantified by ImageJ software and normalized to Gapdh levels. *p<0.05 compared to control, +p<0.05 compared to the cells treated with βNF.

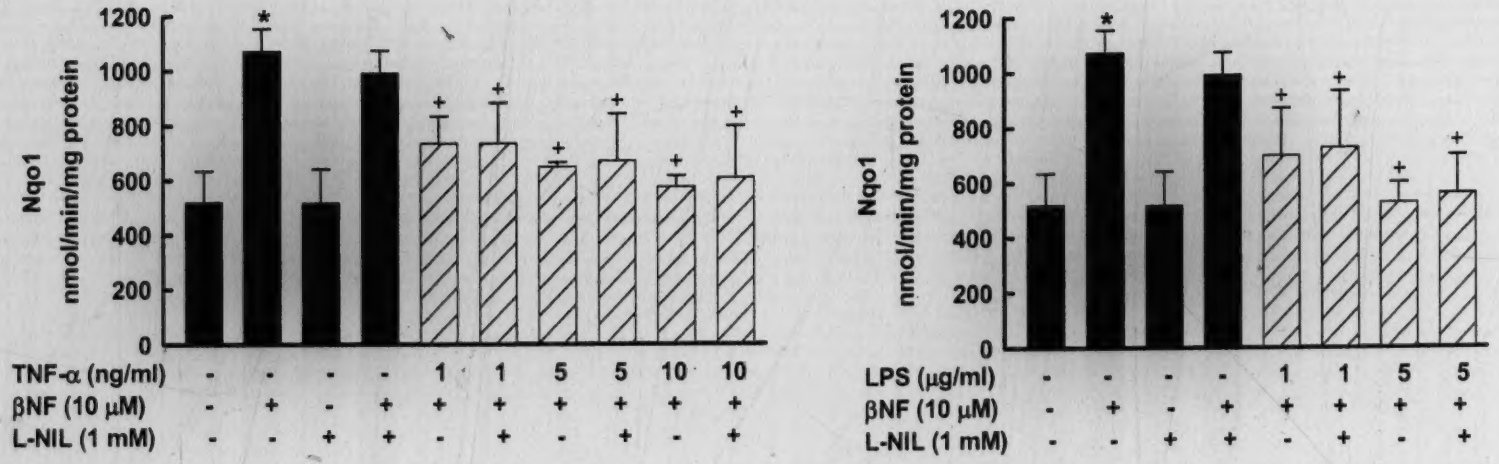

Figure 8.8. Effect of TNF-α and LPS on the βNF-mediated induction of Nqo1 activity in the absence or presence of L-NIL. Cells were treated with βNF (10 μM) and TNF-α (1, 5 and 10 ng/ml) or βNF (10 μM) and LPS (1 and 5 μg/ml) in the absence or presence of L-NIL (1 mM) for 24 h prior to enzyme activity assay (n = 6), respectively. Data are expressed as mean ± SD, *p<0.05 compared to control, +p<0.05 compared to the cells treated with βNF.

Effect of the peroxynitrite decomposer, FeTMPyP on TNF-α and LPS-mediated the down-regulation of inducible expression of Cyp1a1 and Nqo1

To identify whether the decrease in Cyp1a1 and Nqo1 genes by TNF-α and LPS is mediated by peroxynitrite formation, Hepa 1c1c7 cells were treated with peroxynitrite decomposer, FeTMPyP (5 μM), 2 h prior to the treatment of the cells with TNF-α (10 ng/ml) or LPS (5 μg/ml) in the presence of βNF. Our results demonstrate that FeTMPyP did not significantly alter the TNF-α and LPS-mediated decrease in the inducible expression of Cyp1a1 and Nqo1 at mRNA and activity levels (Figure 8.9A, B and 8.10A, B). These results suggest that peroxynitrite is not involved in the down-regulation of *Cyp1a1* and *Nqo1* gene expression by TNF-α and LPS.

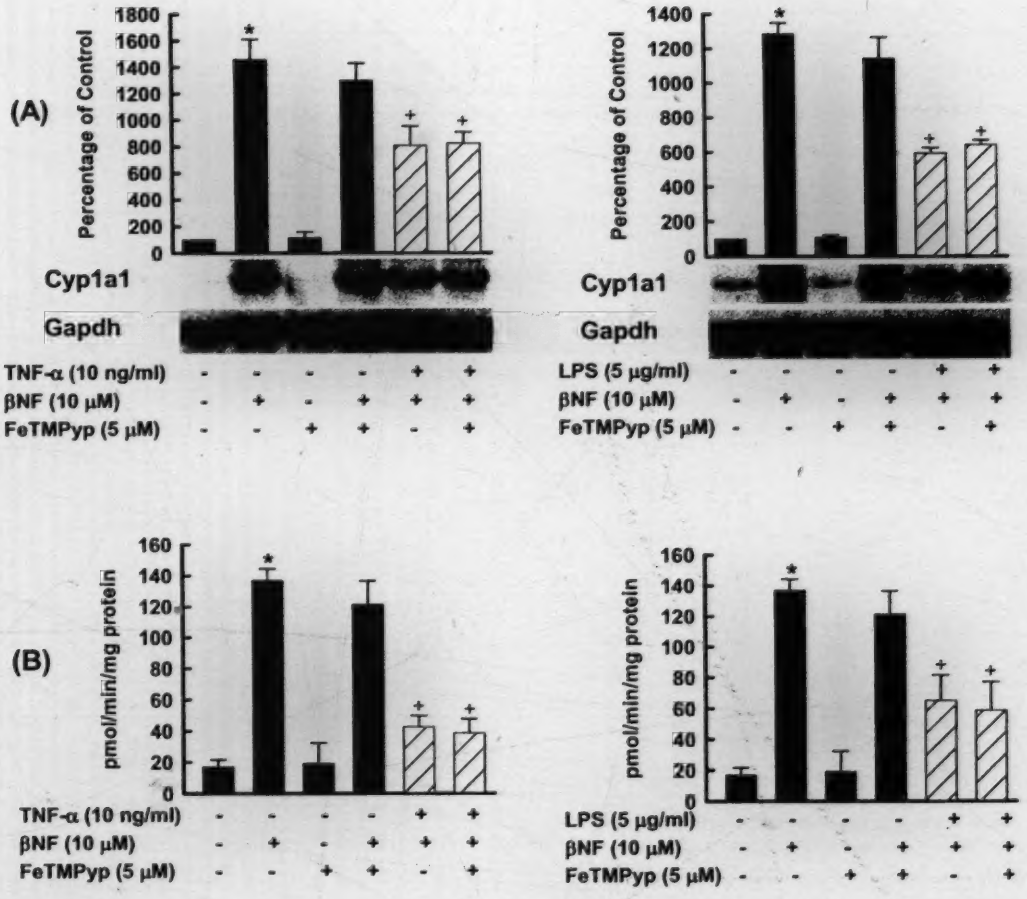

Figure 8.9. Effect of TNF-α and LPS on the βNF-mediated induction of Cyp1a1 mRNA (A) and activity (B) in the absence or presence of FeTMPyP. Cells were treated with βNF (10 μM) and TNF-α (1, 5 and 10 ng/ml) or βNF (10 μM) and LPS (1 and 5 μg/ml) in the absence or presence of FeTMPyP (5 μM) for 6 and 24 h prior to Northern blot analysis (n=3) and enzyme activity assay (n = 8), respectively. Data are expressed as mean ± SD, *p<0.05 compared to control, +p<0.05 compared to the cells treated with βNF.

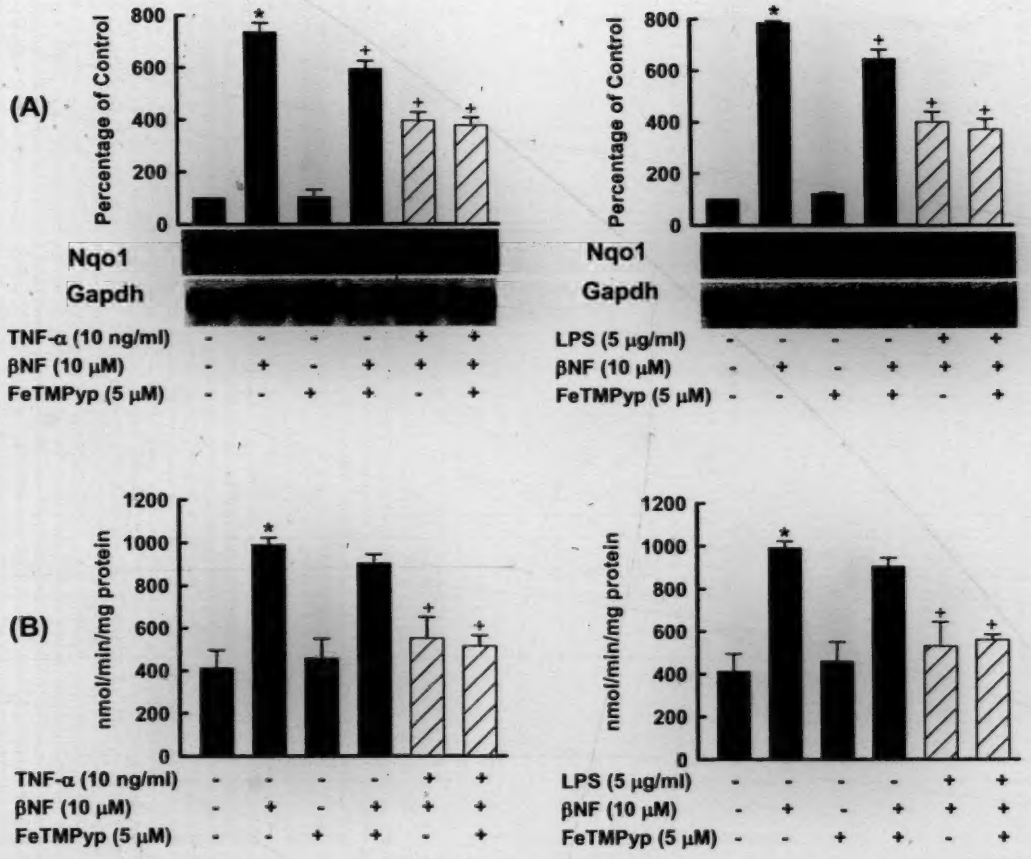

Figure 8.10. Effect of TNF-α and LPS on the βNF-mediated induction of Nqo1 mRNA (A) and activity (B) in the absence or presence of FeTMPyP. Cells were treated with βNF (10 μM) and TNF-α (1, 5 and 10 ng/ml) or βNF (10 μM) and LPS (1 and 5 μg/ml) in the absence or presence of FeTMPyP (5 μM) for 6 and 24 h prior to Northern blot analysis (n = 3) and enzyme activity assay (n=6), respectively. Data are expressed as mean ± SD, *p<0.05 compared to control, +p<0.05 compared to the cells treated with βNF.

Discussion

NO is a highly reactive and diffusible gas that is produced in a number of tissues, and exerts wide range of physiological and pathophysiological effects. NO overproduced by NOS2 has been implicated in a plethora of biologic functions including the drug metabolizing enzymes (Eum et al. 2006). While most of the studies examining the role of NO in the modulation of CYP used an exogenous source for the NO or a pharmacological inhibitor for NOS2, very few studies have examined the role of endogenously produced NO. Investigations of other effects of NO, particularly the inhibition of mitochondrial respiration, revealed that major differences between exogenously applied and endogenously produced NO can be observed (Stadler et al. 1991). In light of these observations in this study we examined the effect of endogenously produced NO in Hepa 1c1c7 cells in response to TNF-α and LPS on the *Cyp1a1* and *Nqo1* gene expression.

Therefore as a first step to determine the role of NO in the down-regulation of AhR-regulated genes during inflammation, we tested the effect of TNF-α and LPS on the expression of NOS2 and NO production in Hepa 1c1c7 cells. Our results show that the incubation of the cells with TNF-α and LPS, cause a concentration-dependent increase in the expression of NOS2 resulting in the release of substantial quantities of NO which was significantly prevented by the NOS2 inhibitor, L-NIL (Figure 8.3 and 8.4). In agreement with our results, it has been reported that TNF-α and LPS are able to induce NO production in cultured rat hepatocytes (Morris and Billiar 1994; Pittner and Spitzer 1992).

In the current study, we provided evidences that the down-regulation of Cyp1a1 in Hepa 1c1c7 cells occurred by a combination of NO-dependent and NO-independent pathways. In support of the role of NO in Cyp1a1 suppression by inflammation, our results demonstrate that the down-regulation of Cyp1a1 by TNF-α and LPS was accompanied with the formation of significant amounts of NO and an increase in NOS2 mRNA expression. In addition, NOS2 inhibitor, L-NIL, inhibited the TNF-α and LPS-mediated production of NO and partially prevented the TNF-α and LPS-mediated decrease in Cyp1a1 at mRNA and activity levels (Figure 8.3, 8.5, and 8.6). In agreement with our results, Stadler et al., reported that in rat hepatocytes, cytokine mixture of IL-1, TNF-α, IFN-γ and LPS caused relatively small changes in the constitutive and inducible levels of Cyp1a1 and Cyp1a2 which was partially prevented by NOS2 inhibitor, N-methyl-L-arginine (Stadler et al. 1994). Recent studies using the isolated perfused rat liver, showed that the effect of NO on the down-regulation of CYP is time-, concentration- and isoenzyme-dependent (Vuppugalla and Mehvar 2004b; Vuppugalla and Mehvar 2004a). On the other hand, the amounts of NO produced by various concentration of LPS was similar while increasing concentrations of LPS produced a progressive decrease in βNF-mediated induction of Cyp1a1 mRNA and activity suggesting that other mediators in addition to NO are involved. Furthermore, partial protection of the TNF-α and LPS-mediated decrease in Cyp1a1 expression by NOS2 inhibitor confirmed the role of other mediators in addition to NO.

NO can inhibit the CYP by two different mechanisms, which are reversible and irreversible mechanisms (Wink et al. 1993). The reversible inhibition is most likely due

to the direct interaction of NO with the iron of the heme prosthetic group (Roberts et al. 1998). In contrast, the irreversible inhibition is due to the formation of peroxynitrite ($ONOO^-$) (Roberts et al. 1998). It is likely that $ONOO^-$ can be generated as a consequence of overproduction of NO and superoxide during inflammatory stimulation (Crow and Beckman 1995). The formation of $ONOO^-$ can initiate a cascade of biochemical events that can disrupt virtually all the cellular components, but is particularly detrimental to the cellular lipids and proteins (Berg et al. 2004). Peroxynitrite is capable of modifying CYP by tyrosine nitration or thiol oxidation (Liaudet et al. 2000). Our results show that FeTMPyP (Hunt et al. 1997), a peroxynitrite decomposer, did not significantly prevent the TNF-α and LPS-mediated down-regulation of Cyp1a1 at both mRNA and activity levels (Figure 8.9).

In vivo, there is contradictory evidence about the role of NO in the LPS-mediated changes to Cyp enzymes. In agreement with our results, a number of studies have shown that LPS-mediated decrease of hepatic Cyp1a and Cyp2b in mice were associated with NOS2 induction and NO overproduction and that the response is prevented by NOS inhibitors (Khatsenko et al. 1998; Khatsenko et al. 1993). In contrary, it has been reported that in NOS2 knockout mice, various Cyp mRNA and proteins could still be depressed by LPS, indicating that inflammation can down-regulate CYP in a NO-independent manner (Sewer et al. 1998; Sewer and Morgan 1998). Two different mechanisms have been proposed to explain such controversy and may be responsible for the down-regulation of CYP following the intraperitoneal administration of LPS in rats (Ferrari et al. 2001; Morgan et al. 2002). At high concentrations of LPS, an NO-dependent decrease in CYP

occurs via the production of NO-derived products, such as peroxynitrite, while at low concentrations of LPS, an NO-independent decrease in CYP occurs via loss in transcription and mRNA production.

Several mechanisms may have contributed to the suppression of Cyp1a1 by TNF-α or LPS. NF-kB, a well-studied transcription factor, shows inhibitory interaction with AhR signalling pathway and is activated by TNF-α or LPS. (Ghosh et al. 1998; Ke et al. 2001). It has been reported that, NO enhanced the NF-kB activity in TNF-α-stimulated endothelial cells (Umansky et al. 1998). In addition, L-NIL, a NOS2 inhibitor, prevented LPS-induced activation of NF-kB in mouse peritoneal and primary rat alveolar macrophages, suggesting that NO can directly increase the NF-kB activity (Kang et al. 2000). Taken together, theses data suggest that the partial protective effect of L-NIL on TNF-α- or LPS-mediated down-regulation of *Cyp1a1* gene expression is due to the inhibition NF-kB activity.

Another potential mediator that may be involved in the suppression of AhR-regulated genes by TNF-α or LPS is reactive oxygen species (ROS). We have previously shown that TNF-α and LPS significantly increase the ROS production in Hepa 1c1c7 cells (Gharavi and El-Kadi 2005b). ROS such as H_2O_2 may inactivate CYP1A1 by direct interaction with the CYP1A1-associated Fe^{2+} (Karuzina and Archakov 1994; Takemura et al. 1999). However, Morel et al., have reported that in HepG2 cells, *CYP1A1* promoter activity, which is a target of nuclear factor I/CCAAT box transcription factor (NFI/CTF-1) is suppressed by oxidative stimuli through suppression of NFI/CTF-1 (Morel et al.

2000). A cysteine residue within the *trans*-activating domain of NFI/CTF-1 is the regulatory target of H_2O_2 (Morel et al. 2000). Suppression of *CYP1A1* promoter activity by TNF-α was also dependent on NF1 binding site, suggesting that TNF-α down-regulates CYP1A1 expression through a redox mechanism involving NFI (Morel and Barouki 1998). On the other hand, ROS can also activate NF-kB. Based on these results, ROS production mediated by TNF-α and LPS can suppress CYP1A1 possibly through the activation of NF-kB or inactivation of NFI/CTF-1 (Flohe et al. 1997; Janssen-Heininger et al. 2000; Morel et al. 2000; Schreck and Baeuerle 1991).

With respect to phase II metabolizing enzymes, the regulation of *Nqo1* gene in different animal tissues and cell lines have shown a complex molecular pathway. Analysis of the 5'-flanking region of *Nqo1* gene demonstrated that the presence of two distinct responsive elements, namely the XRE and the antioxidant responsive element (ARE), which are closely located to each other, mediate the transcriptional activation of *Nqo1* gene (Rushmore and Kong 2002). Independent induction of these genes by ARE inducers has been shown to be mediated by the activation of a labile transcriptional factor, nuclear factor erythroid 2-related factor-2 (Nrf2) (Nioi and Hayes 2004). Nrf2 is retained in the cytoplasm by binding to its inhibitory protein, Kelch-like ECH associating protein 1 (Keap1) (Nioi and Hayes 2004). Upon activation, Nrf2 dissociates from Keap1 protein and then translocates to the nucleus, where it dimerizes with a small Maf protein. The Nrf2-Maf complex then binds to the ARE consensus sequence located in the promoter region of *Nqo1* gene, resulting in the initiation of the transcription process (Chen et al. 1994; Jaiswal 2004; Nioi and Hayes 2004).

We previously showed that TNF-α and LPS down-regulated the Nqo1 expression through XRE- but not through ARE-dependent pathway (Gharavi and El-Kadi 2005b). In the present study we showed that neither L-NIL nor FeTMPyP alter the down-regulation of Nqo1 mediated by TNF-α and LPS at mRNA and activity levels (Fig 8.7, 8.8, and 8.10), suggesting that NO and peroxynitrite do not play a role in this down-regulation.

Taken together, the evidences provided in this study suggest that the induction of NOS2 and the resulting production of NO, but not peroxynitrite, play a role in mediating the effects of TNF-α and LPS on Cyp1a1 expression. However, the down-regulation of Nqo1 by TNF-α and LPS is not mediated by NO or peroxynitrite.

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PART III

GENERAL DISCUSSION AND CONCLUSION

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The AhR has occupied the attention of scientists for over two decades. Interest arose from the early observation that this soluble protein played key roles in the toxicity and metabolic response to polycyclic aromatic hydrocarbons and halogenated aryl hydrocarbons (Schmidt and Bradfield 1996). It has been recognized that the AhR mediates the induction of various xenobiotic metabolizing enzymes, including four phase I enzymes (CYP1A1, CYP1A2, CYP1B1, and CYP2S1) and four phase II xenobiotic metabolizing enzymes, including NQO1, GSTA1, ALDH-3, and UGT1A6 (Nebert et al. 1993; Rivera et al. 2002). These enzymes are distributed in various tissues such as liver and gastrointestinal tract that play an important role in first-pass metabolism and drug metabolism. Among AhR-regulated genes, CYP1A1, CYP1A2 and CYP2B1 are capable of producing epoxides and dihydrodiol epoxides from aromatic and halogenated hydrocarbons (Schrenk 1998). These toxic metabolites have been shown to be involved in the mediation of a broad range of toxic responses such as immune suppression and carcinogenesis (Poland and Knutson 1982). However, the mechanisms for these AhR-mediated pathophysiological conditions remain unknown.

In humans and experimental animals, activities of CYPs are usually down-regulated by infections and inflammation, although some are induced or unaffected (Morgan 1997). CYPs are responsible for the bioactivation and/or detoxification and clearance of many drugs and toxicants, thereby playing a critical role in the pharmacological and toxicological effects of many compounds. Suppression of CYP

during an inflammatory response may result in reduced therapeutic or toxic effects of drugs that are metabolized to pharmacologically or toxicologically active compounds or can lead to increased clinical toxicity of drugs with low therapeutic indices (Morgan 1997). These changes have been linked to increased serum concentrations of proinflammatory cytokines (Bleau et al. 2001; El-Kadi and du Souich 1998; El-Kadi et al. 1997). Although much has been learned concerning the effects of inflammation on CYPs such as CYP1A1, there is relatively little information concerning the underlying mechanisms and the regulation of other AhR-regulated genes such as NQO1 and GSTA1 during inflammation, and this area requires much more study to determine the molecular mechanisms involved.

To establish the cross-talk between AhR and inflammation on AhR-regulated genes, we determined the effect of TNF-α and LPS on the mRNA and activity levels of Cyp1a1, Nqo1, and Gsta1 in murine hepatoma Hepa 1c1c7 cells (WT). The availability of mutant Hepa 1c1c7 cell lines deficient in AhR and ARNT allowed us to investigate the importance of the presence of both partners of a heterodimeric transcription factor, AhR and ARNT. In the current research project, the role of both AhR and ARNT in suppression of AhR-regulated genes by TNF-α or LPS is demonstrated based on the finding that TNF-α or LPS down-regulated the βNF-mediated induction of Cyp1a1, Nqo1 and Gsta1 in WT but not in AhR-deficient (C12) and ARNT-deficient (C4) cells. Further evidence to support this conclusion comes from the observation that TNF-α or LPS did not significantly affect the tBHQ-mediated induction of Nqo1 and Gsta1 at both activity and mRNA levels. The changes at the enzyme activity were correlated with the

changes in mRNA expression, suggesting the modulation of Cyp1a1, Nqo1, and Gsta1 by TNF-α or LPS is through a pre-transcriptional mechanism. However, some other studies have reported that the down-regulation of CYP1A1 by other cytokines, including IL-1 and IFNs, is due to a post-transcriptional mechanism such as RNA turnover. For example, in rat hepatocytes treated with IL-1, CYP1A1 mRNA levels declined compared to their corresponding control (Barker et al. 1992). Also the interferon-mediated loss of CYP1A1 in rat involves an increase in mRNA degradation (Delaporte and Renton 1997).

Several mechanisms may be involved in the suppression of AhR-regulated genes by TNF-α or LPS. Cross-talk between the AhR and the NF-κB which is activated by TNF-α or LPS suggests that NF-κB may play a direct role in mediating the suppression of CYP1A1 expression by inflammatory agents (Tian et al. 1999). It has been found that the nuclear receptor coactivators CBP, p300/CBP and SRC-1 and the corepressor silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) associate with an active subunit of NF-κB, AhR, and ARNT, suggesting that coregulator proteins may contribute to the mediation of the inhibitory interaction between AhR and NF-κB (Ke et al. 2001; Kobayashi et al. 1997; Kumar et al. 1999; Lee et al. 2000; Nguyen et al. 1999; Perkins et al. 1997). Competition between AhR/ARNT complexes and NF-κB for binding of coregulator proteins may alter the level of expression of AhR-regulated genes.

Some studies have reported the contribution of phosphorylation pathways in the suppression of CYPs by LPS or proinflammatory cytokines. For examples, various protein kinase inhibitors such as inhibitors of JAK, ERK1/2, p42/44 MAPK, and PKC

have shown to partially prevent the inhibition of CYP1A1, 1A2, and 3A6 activities and reduction of total CYP content in cultured rabbit hepatocytes produced by cytokines such as IL-6 (Levitchi et al. 2004). In addition, LPS can suppress Cyp1a1 mRNA expression directly through the activation of the MAPK (ERKs, JNKs, and p38) pathway (Guha and Mackman 2001).

Several studies have shown that the production of a number of ROS during infection or inflammation is responsible for the down-regulation of CYP (Chen et al. 2005; El-Kadi et al. 2000; El-Kadi et al. 1997; Xu et al. 2004). There is evidence that ROS may directly or indirectly contribute to the suppression of CYP such as CYP1A1 and 1A2. ROS such as H_2O_2 may directly interact with the CYP-associated Fe^{2+}, results in heme destruction followed by CYP inactivation (Karuzina and Archakov 1994). It can also activate phosphorylation signalling pathways such as MAPK and protein kinase A and C, which are involved in the phosphorulation of CYP, and indirectly leads to inactivate the enzyme (Bae et al. 1997; Boyer et al. 1995; Goldstone and Hunt 1997; Lowe et al. 1998; Suzuki et al. 1997). In the research project reported in this thesis, we have shown that the decrease in AhR-regulated genes by TNF-α or LPS was associated with an increase in ROS production. The increase in ROS production induced by TNF-α or LPS was significantly prevented by the phenolic antioxidant, tBHQ, while it was not significantly affected by the AhR antagonist, αNF. These results show that the protective effect of tBHQ on Cyp1a1, Nqo1 and Gsta1 is due to the inhibition of ROS production and the AhR may not be involved in the production of ROS by TNF-α or LPS. In support of with our results, it has been reported that tBHQ inhibits the TNF-α- or LPS-induced

ROS production (Ma and Kinneer 2002). ROS can also alter the activity of transcription factors such as NF-κB, activator protein-1, and NFI/CTF-1, which are involved in the modulation of CYP expression during inflammatory stimuli (Abdel-Razzak et al. 1994; Janssen-Heininger et al. 2000; Morel et al. 2000; Nebert et al. 1993). It has been shown that either ROS-mediated activation of NF-κB or TNF-α- and LPS-mediated suppression of CYP1A1 expression prevented by antioxidants (Janssen-Heininger et al. 2000). It has also been reported that tBHQ prevents the activation of NF-κB induced by TNF-α or LPS (Ma and Kinneer 2002). These results suggest that ROS is involved the TNF-α- and LPS-mediated suppression of the CYP1A1 expression, possibly through the activation of NF-κB activity and the protective effect of tBHQ on suppression of AhR-regulated genes by TNF-α or LPS may be due to the inhibition of NF-κB activity (Ma and Kinneer 2002). *CYP1A1* gene is also down-regulated by oxidative stimuli through modulation of the binding of NFI/CTF-1 to the *CYP1A1* promoter (Morel and Barouki 1998; Morel et al. 2000).

Meanwhile, other aspects of our data on the effects of tBHQ on the Cyp1a1 induction are also worth noting. Antioxidants like tBHQ are believed to represent monofunctional inducers, which selectively induce phase II metabolizing enzymes via an AhR-independent pathway. However, our results have shown that tBHQ acts as an AhR ligand and induces phase I metabolizing enzyme, Cyp1a1 in Hepa 1c1c7 cells. We demonstrated that a significant concentration-dependent increase in Cyp1a1 mRNA, protein and activity occurred after treatment of Hepa 1c1c7 cells with tBHQ. The increase in mRNA was apparent 3 h after treatment. The RNA polymerase inhibitor,

actinomycin D, completely blocked the Cyp1a1 induction by tBHQ, indicating a requirement of *de novo* RNA synthesis through transcriptional activation. The protein synthesis inhibitor cycloheximide superinduced the tBHQ-mediated induction of Cyp1a1 mRNA and completely prevented the increase in Cyp1a1 activity, indicating that the induction of enzyme activity by tBHQ is dependent on *de novo* protein synthesis. In addition, the AhR antagonist, resveratrol, inhibited the increase in Cyp1a1 activity by tBHQ. Gel electrophoretic mobility shift assays showed that tBHQ causes activation or transformation of the AhR in nuclear extracts, indicating that AhR-dependent mechanisms contributed to the Cyp1a1 induction. Similar to Hepa 1c1c7 cells, tBHQ caused a concentration-dependent increase in CYP1A1 at the mRNA and activity levels in human HepG2 cells. This is the first demonstration that the phenolic antioxidant, tBHQ, can directly induce *Cyp1a1* gene expression through AhR-dependent pathway. Taken together, tBHQ can be categorized as bifunctional inducer, which induces both, phase I and II metabolizing enzymes through AhR- and antioxidant response element (ARE)-dependent mechanisms.

Suppression of AhR-regulated genes by TNF-α or LPS may modulate by production of another potential mediator such as NO. Activation of different cell types such as macrophages, endothelial cells, fibroblasts and hepatocytes by proinflammatory cytokines results in the induction of NOS2 expression followed by NO production (Xie et al. 1992). Much controversy has surrounded the role of NO as a mediator in the down-regulation of CYP expression during inflammation. Although a number of studies have shown that inflammation-mediated suppression in hepatic CYP correlate with NO

production and that response is blocked by NOS2 inhibitors, others showed that NOS2 inhibition has no effect (Carlson and Billings 1996; Khatsenko and Kikkawa 1997; Khatsenko et al. 1998; Khatsenko et al. 1993; Minamiyama et al. 2001; Minamiyama et al. 1997; Sewer et al. 1998; Sewer and Morgan 1998; Shimamoto et al. 1998). For example, in NOS2 knockout mice, various CYP mRNA and proteins could still be depressed by LPS, indicating that inflammation can down-regulate CYP in a NO-independent manner (Sewer et al. 1998; Sewer and Morgan 1998). In pig hepatocytes, Monshouwer et al. also demonstrated that complete inhibition of NOS had no effect on the loss of CYP in response to IL-1, IL-6, or TNF-α (Monshouwer et al. 1996). In rat hepatocytes cultured on Matrigel, CYP2C11 down-regulation in response to cytokines could not be altered after complete inhibition of NO production (Sewer and Morgan 1997). Also in mice treated with N^{ω}-nitro-L-arginine the decrease in total CYP or Cyp1a or Cyp2e1 activity caused by an IFN inducer did not change (Hodgson and Renton 1995).

In this research project, our results demonstrated that a combination of NO-dependent and NO-independent pathways plays a role in the down-regulation of Cyp1a1 in Hepa 1c1c7 cells. In support of the role of NO in TNF-α- or LPS-mediated suppression of CYP1A1, we found that suppression of Cyp1a1 was accompanied with an increase in NOS2 mRNA expression followed by the production of NO which was inhibited with the NOS2 inhibitor, L-NIL. In addition, L-NIL partially prevented the TNF-α- and LPS-mediated down-regulation of Cyp1a1 at both mRNA and activity levels. In support of our observation, studies in rat hepatocytes have shown that changes in the constitutive and inducible expression of Cyp1a1 and 1a2 caused by a mixture of IL-1, TNF-α, IFN-γ and

LPS was also partially prevented by NOS2 inhibitor, N-methyl-L-arginine (Stadler et al. 1994). On the other hand, other evidence provided in our study confirms the role of other mediators in addition to NO. For example, although increasing concentrations of LPS produced a progressive decrease in βNF-mediated induction of Cyp1a1 mRNA and activity the amounts of NO produced by various concentrations of LPS was similar. Furthermore, the TNF-α- and LPS-mediated decrease in Cyp1a1 expression was partially protected by L-NIL. It is known that NO can inactivate CYP by reversible and irreversible mechanisms (Wink et al. 1993). The reversible inhibition is likely due to the direct interaction of NO with the iron of the heme group (Roberts et al. 1998). However, the irreversible inhibition is due to the formation of peroxynitrite (Roberts et al. 1998), which can inactivate CYP by tyrosine nitration or thiol oxidation (Liaudet et al. 2000). In this study, based on the results that a peroxynitrite decomposer, FeTMPyP did not significantly prevent suppression of CYP1a1 mRNA and activity mediated by TNF-α and LPS we suggested that peroxynitrite does not contribute to the Cyp1a1 suppression.

With respect to phase II metabolizing enzymes, our results have shown that TNF-α and LPS down-regulated Nqo1 expression through AhR but not ARE-dependent pathway. Also the down-regulation of Nqo1 mediated by TNF-α and LPS at both mRNA and activity levels were not changed by neither L-NIL nor FeTMPyP, suggesting that NO and peroxynitrite may not be involved in Nqo1 down-regulation.

Taken together, the results obtained from this research project allow us to conclude that the down-regulation of AhR-regulated genes by TNF-α and LPS is

dependent on the presence of both heterodimeric transcription factors, AhR and ARNT. Furthermore, association of this down-regulation with an increase in ROS production reveals that ROS may directly or indirectly be involved in down-regulation of AhR-regulated genes. On the other hand, NO, but not peroxynitrite, may be involved in TNF-α- and LPS-mediated down-regulation of Cyp1a1 without affecting the down-regulation of Nqo1.

Future studies:

The results obtained from the present research project also raise questions that may be answered by conducting studies:

1. to assess the effect of TNF-α or LPS on mRNA and protein stabilities of AhR-regulated genes.
2. to examine the effect of TNF-α or LPS on the transcription rate of AhR-regulated genes.
3. to investigate the direct role of redox-sensitive signalling pathways involving transcription factors such as NF-kB and AP-1 in the down-regulation of AhR-regulated genes during inflammation.
4. to examine the role of coregulatory proteins such as coactivator and corepressor proteins in the cross-talk between AhR and NF-kB/AP-1 signalling pathways in the down-regulation of AhR-regulated genes.
5. to characterize the role of phosphorylation and MAPK signalling pathways in the modulation of AhR-regulated genes during inflammation.

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